
CIRCADIAN MODULATION OF OSMOREGULATED FIRING IN RAT SUPRAOPTIC NUCLEUS NEURONS

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Short title: **Clock control of SON neurons**

Abstract

The antidiuretic hormone vasopressin (VP) promotes water reabsorption from the kidney and levels of circulating VP are normally related linearly to plasma osmolality to maintain the latter close to a predetermined set point. Interestingly, VP levels rise also in the absence of an increase in osmolality during late sleep in various mammals including rats and humans. This circadian rhythm is functionally important because the absence of a late night VP surge results in polyuria and disrupts sleep in humans. Previous work has indicated that the VP surge may be caused by a facilitation of the central processes that mediate the osmotic control of VP release and the mechanism by which this occurs was recently studied in angled slices of rat hypothalamus that preserve intact network interactions between the suprachiasmatic nucleus (SCN, the biological clock), the organum vasculosum lamina terminalis (OVLT, the central osmosensory nucleus) and the supraoptic nucleus (SON, which contains VP releasing neurohypophysial neurons). These studies confirmed that the electrical activity of SCN clock neurons is higher during the middle sleep period (MSP) than during the late sleep period (LSP). Moreover, they revealed that the excitation of SON neurons caused by hyperosmotic stimulation of the OVLT was greater during the LSP than during the MSP. Activation of clock neurons by repetitive electrical stimulation, or by injection of glutamate into the SCN, caused a presynaptic inhibition of glutamatergic synapses made between the axon terminals of OVLT neurons and SON neurons. Consistent with this effect, activation of clock neurons with glutamate also reduced the excitation of SON neurons caused by hyperosmotic stimulation of the OVLT. These results suggest that clock neurons in the SCN can mediate an increase in VP release through a disinhibition of excitatory synapses between the OVLT and the SON during the LSP.

Key words: Suprachiasmatic nucleus ; vasopressin ; supraoptic nucleus ; OVLT ; organum vasculosum lamina terminalis ; presynaptic inhibition ; circadian ; osmoregulation.

Vasopressin (VP) and systemic osmoregulation.

Systemic osmoregulation is a vital homeostatic process because acute deviations in extracellular fluid osmolality (moles of solutes per kilogram of solution) can cause significant cellular shrinking or swelling and thus damage tissues and organs [1]. Being encased in a rigid cranium, the mammalian brain is particularly vulnerable to osmotic insults. Although changes in plasma osmolality smaller than ±10% (i.e. ±30 mosmol/kg) are usually innocuous, rapid osmotic perturbations of greater magnitude can induce symptoms progressing from headache and mental confusion, to seizures, coma and death [2-4]. Acute changes in plasma osmolality are caused by quantitative differences in the gain or loss of salt and water, and therefore occur as a normal part of every day life. Drinking a liter of water over a few minutes, for example, lowers plasma osmolality by 2% within 30 minutes [5]. In healthy individuals osmotic fluctuations normally induce compensatory changes in salt and water intake, as well as rapid changes in renal sodium excretion (natriuresis) and water excretion (diuresis) so that the average plasma osmolality stays close to a species-specific set-point [6-8].

Centrally-mediated adjustments in the release of vasopressin (VP) from the neurohypophysis provide one of the most rapid and effective mechanisms available for systemic osmoregulation in all mammals. VP (antidiuretic hormone) is synthesized in the somata of magnocellular neurosecretory cells (MNCs) located in the supraoptic (SON) and paraventricular nuclei. Notably these nuclei also contain another population of MNCs synthesizing oxytocin, a hormone that plays a key role in lactation and parturition [9]. The MNCs project their axons into the neurohypophysis, where VP and oxytocin are released into the general circulation via neurosecretory endings apposed to fenestrated capillaries. Previous work has established that hormone release from the axon terminals of MNCs is triggered by calcium-dependent exocytosis subsequent to the opening of voltage-gated calcium channels upon arrival of action potentials

[10-12]. Accordingly, hormone release occurs in proportion with the frequency of action potential discharge by MNCs [11, 13] and the central control of VP and oxytocin secretion from the neurohypophysis is achieved by regulating the excitability of MNCs [7, 9, 14].

Interestingly, oxytocin is a potent stimulator of natriuresis in rats [15-17], and the osmotic control of oxytocin releasing MNCs has been shown to play a significant role in osmoregulation in this species. Most electrophysiological studies concerning the osmotic regulation of MNCs performed in rats have not systematically identified the neurons studied and rely on the assumption that that both types of cells are regulated by equivalent mechanisms. Evidently this assumption remains to be verified. In the remainder of this article we uniquely refer to VP to reflect the fact that this is the key neurohypophysial hormone that contributes to osmoregulation in all mammals. We also simply refer to "MNCs" to reflect the fact that the cells investigated in our studies were not specifically identified as VP or oxytocin containing.

Osmotic control of MNCs.

Changes in VP release in response to fluctuations in plasma osmolality are mediated through a combination of mechanisms. First, glial cells surrounding MNCs have been found to release taurine as an inverse function of fluid osmolality [18]. Because taurine is an agonist of the chloride-permeable glycine receptor, this effect promotes membrane hyperpolarization and inhibition of MNCs under hypoosmotic conditions [19]. Second, MNCs have been shown to express a shrinking-activated cation channel encoded by the transient receptor potential vanilloid type 1 (*trpv1*) gene [20]. Activation of these channels during hypertonicity promotes membrane depolarization and neuronal excitation [21, 22]. Third, MNCs receive afferent inputs from osmosensitive neurons located in the OVLT (organum vasculosum laminae terminalis), the brain's primary osmoreceptor area [23]. Previous studies have shown that OVLT neurons project

to the SON [24, 25], where they make monosynaptic glutamatergic contacts with MNCs [26-28]. The firing rate of osmosensitive OVLT neurons declines during hypotonicity and increases during hypertonicity [29, 30]. Moreover, local osmotic stimulation of the OVLT has been shown to cause proportional changes in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) or potentials (sEPSPs) mediated by AMPA receptors [28]. In contrast, osmotic stimulation of the OVLT does not affect the frequency of spontaneous inhibitory postsynaptic potentials (sIPSPs) in SON neurons [31]. These observations suggest that osmosensitive OVLT neurons use glutamate as a transmitter and mediate the osmotic control of MNCs via proportional changes in synaptic excitation [31-33].

Although all three of the above mechanisms contribute to the osmotic control of MNCs, experiments *in vivo* have shown that lesions of the OVLT profoundly inhibit the activity of MNCs [34] and severely impair the osmotic control of VP release [35, 36]. The excitatory synaptic drive provided by OVLT neurons therefore appears to be an essential requirement for the generation of action potentials and neurosecretion by MNCs *in situ*. Indeed, osmotically-induced changes in the firing rate of MNCs in hypothalamic explants have been found to correlate with sEPSP frequency [31], indicating that the modulation of OVLT-mediated osmosensory synaptic drive plays a key role in the osmotic control of VP release.

Non-osmotic modulation of VP commonly involves changes in osmoregulatory gain.

Although VP secretion is exquisitely sensitive to changes in plasma osmolality, release of this hormone is also modulated by other physiological parameters to mediate adaptive changes in diuresis. For example, MNCs can be excited by local increases in temperature and this effect appears to contribute to VP release during hyperthermia caused by exercise or exposure to ambient heat [37-39]. The release of VP during systemic hyperthermia is an anticipatory

phenomenon, because it can be detected in the absence of changes in plasma osmolality, yet it presumably serves to minimize the eventual development of hypertonicity caused by the loss of body water through sweat produced for thermoregulatory cooling (sweat is a hypotonic fluid [40]). Studies in humans have indicated that increases in core body temperature also enhance the VP response to increases in plasma osmolality [41], suggesting that the thermal stimulation of VP release may in fact be mediated by a modulation of the mechanisms responsible for the osmotic control of MNCs. Indeed, thermal and osmotic stimuli have been shown to induce an additive activation of OVLT and SON neurons as measured by expression of the immediate early gene *c-fos* [42].

The secretion of VP is also markedly enhanced by isotonic hypovolemia (e.g. during hemorrhage) [6, 8, 43]. Together with the intake of salt and water this response is beneficial because it promotes a replenishment of extracellular fluid volume [44]. Conversely, VP release is suppressed by hypervolemia which enhances diuresis to eliminate excess fluid volume [6, 8, 43]. Interestingly, previous work has shown that changes in VP release provoked by changes in blood volume are tightly linked to changes in the slope of the relationship between plasma osmolality and circulating VP concentration [6, 8, 43]. The influence of extracellular fluid volume on VP may therefore also be mediated by a modulation of the mechanisms responsible for the osmotic control of MNCs.

Circadian control of VP involves modulation of osmoregulatory gain.

In many species of mammals [45-47], including humans [48, 49], a progressive increase in circulating VP concentration is observed during the last part of the sleep period, reaching a peak around wake time. This increase in VP secretion occurs in the absence of a rise in plasma

osmolality, which in fact appears to decline in concert with the rise in hormone level [46, 47]. Accordingly, the ratio of plasma VP concentration over osmolality is significantly higher at the end of the sleep period than during other parts of the day [46]. This observation indicates that the relation between plasma VP and plasma osmolality is either shifted toward lower values of plasma osmolality, or that the slope of the relation is increased during the late sleep period (LSP). Although additional work is required to resolve the exact nature of this phenomenon, these results indicate that osmotically-induced VP release may be facilitated during the LSP.

The increase in antidiuretic activity associated with the rise in plasma VP during the LSP plays a significant physiological role because humans lacking this rhythm suffer from nocturnal polyuria and experience disrupted sleep [50-52]. As such, the surge in VP release during the LSP epitomizes the general importance of circadian rhythms for homeostasis. In this case the rise in VP levels serves to protect the body from dehydration at a time when evaporative water loss associated with breathing is not compensated for by water intake, and absence of this rhythm yields a clear and predictable pathological consequence.

Thus three of the most important non-osmotic stimuli that can modulate VP release (plasma volume, body temperature and circadian time) appear to do so through a modulation of the mechanisms that mediate the osmotic control of MNCs. In the remainder of this article we will review recent findings indicating that "clock" neurons in the suprachiasmatic nucleus (SCN) can modulate the strength of excitatory synapses between OVLT neurons and MNCs to mediate circadian changes in the osmotic regulation of action potential firing in these cells.

SCN clock neurons project to VP neurons in the SON.

It is well established that the body's master biological clock resides in the SCN [53-57] and recent molecular and genetic studies have shown that reciprocal and time-locked interactions in the transcription of specific genes can mediate cell-autonomous changes in protein expression with a periodicity of ~24 hrs [57-62]. But cell-autonomous gene regulation mechanisms represent only part of the mammalian clock system [63] and many important issues remain unresolved. For example, the exact mechanisms by which axonal projections from retinal ganglion cells entrain the cycles in biochemical and electrical activity of SCN neurons to the local solar cycle remain unclear. Similarly, the mechanisms by which SCN *"clock"* neurons display coordinated circadian changes in action potential firing rate are not known. Likewise, very little is know about how the SCN coordinates so-called peripheral clocks [64, 65], or how it imposes circadian cycles on behaviour and physiological parameters. SCN transplantation experiments have suggested that diffusible substances released by the SCN may mediate some circadian rhythms [66]. However SCN clock neurons send efferent axonal projections to widespread areas of the central nervous system [67-69] and it is likely that many centrally-regulated circadian rhythms rely on activity-dependent release of neurotransmitters or modulators by the axon terminals of SCN neurons in specific target structures.

Previous studies have shown that SCN neurons send functional axonal projections to MNCs in the SON (e.g. [56, 70, 71]). Indeed, a proportion of SCN neurons can be retrogradely labelled by injection of fluorescent microspheres into the SON (Figure 1A,B), and SCN neurons can be antidromically activated by electrical stimulation of the SON *in vitro* (Figure 1C) and *in vivo* [72, 73]. Moreover, application of the lipophilic membrane stain diI to the SCN causes an orthograde labelling of axons that make intimate contacts with neurons in the SON (Figure 1D,E). Finally, electrical stimulation of the SCN has been shown to induce mixed excitatory and inhibitory synaptic responses in SON neurons (e.g. Figure 1F) [28, 71], suggesting that

GABAergic and glutamatergic neurons located in the SCN project into the SON. A vast number of peptides and other neurotransmitters are expressed in SCN clock neurons [54, 74] and are therefore candidates for the circadian modulation of MNCs.

(Figure 1 near here)

Electrophysiological recordings from SCN clock neurons of nocturnal rodents *in vivo* and *in vitro* have shown that these cells are electrically inactive during the dark phase (i.e. during the wake period), but that their action potential firing rate rises to a peak during the middle of the light phase and then progressively declines to zero by wake time [55, 75]. Thus the firing rate of clock neurons is declining at a time when the osmotic control of MNCs is becoming sensitized. This observation suggests that SCN clock neurons might normally exert an activity-dependent inhibitory influence on MNCs, and that this effect becomes progressively removed during the LSP. While this notion is consistent with the fact that most SCN neurons express the inhibitory neurotransmitter GABA (γ-amino butyric acid) [64, 76, 77] and the existence of inhibitory connections between SCN and SON neurons (Fig. 1F; [28, 71]) it is difficult to imagine how the simple addition or removal of a superimposed inhibitory input could selectively modulate the osmotic control of MNCs. Since glutamatergic osmosensory afferents from the OVLT play a key role in regulating the activity of MNCs and VP secretion, we recently hypothesized and demonstrated that SCN neurons can regulate the osmoresponsiveness of VP neurons by modulating the strength of the excitatory connection between OVLT neurons and MNCs in the rat SON via an effect that is independent of GABA receptors.

Circadian timing is maintained in acute hypothalamic slices

To investigate this question, we used an acute slice preparation of adult rat hypothalamus that preserves network connectivity between neurons in the SCN, OVLT and SON [28]. Because of the hypothesis stated above, the perfusing solution contained bicuculline to remove a possible contribution of $GABA_A$ receptors. Moreover, to ensure that any circadian timing differences observed were not related to the amount of time the slices had been maintained *in vitro*, all slices were prepared a consistent amount of time before the beginning of the recording sessions [70] and different slices were prepared for recording sessions during the subjective LSP and MSP. Previous work has shown that circadian changes in the firing rate of SCN neurons are maintained in hypothalamic slices *in vitro* [78]. Extracellular single unit recordings of firing rate confirmed that subjective circadian time is also maintained in our preparation. In agreement with previous studies [78, 79], the mean firing rate of SCN neurons recorded during the MSP (3.8 ± 0.7 Hz, n = 17) was significantly higher than during the LSP (1.6 ± 0.3 Hz, n = 20).

To determine if osmosensory information detected by OVLT neurons was relayed more effectively during the LSP than during the MSP, we compared the effect of applying a fixed hyperosmotic stimulus to the OVLT (local puffing of a small amount of ACSF supplemented with 55 mM mannitol) at different circadian times. The effects of hyperosmotic stimulation on action potential firing rate and sEPSC frequency were both significantly greater when evoked during the LSP than during the MSP. Specifically, the frequency of action potentials and sEPSCs respectively increased by 22.2 ± 6.9% and 36.2 ± 8.7% during the MSP, compared with 115.9 ± 39.9% (P = 0.03) and 95.7 ± 27.7% (P = 0.03) during the LSP (values are means ± s.e.m. of the average percent changes observed in each cell).

To determine if the higher firing rate of SCN neurons is a causal factor in the effectiveness of osmosensory signalling, we examined if excitation of SCN neurons by local application of glutamate could attenuate the responses induced in MNCs by a hyperosmotic

stimulus applied to the OVLT. As illustrated in figure 2, application of glutamate over the SCN significantly reduced the relative increase in sEPSC frequency and excitation of MNCs caused by delivery of a hyperosmotic stimulus to the OVLT. Thus circadian differences in the firing rate of SCN neurons and osmoregulated control of MNCs are maintained in acute hypothalamic slices, and experimentally-induced excitation of SCN neurons causes a reduction in osmosensory signalling between OVLT neurons and MNCs.

(Figure 2 near here)

Clock neurons cause a presynaptic silencing of osmosensory afferents

To examine if SCN clock neurons regulate the efficacy of osmosensory signalling through a modulation of synaptic strength at glutamatergic OVLT→SON synapses, we first examined the effect of activating SCN neurons on EPSCs evoked in SON MNCs by electrical stimulation of the OVLT. As shown in figure 3, we found that repetitive electrical stimulation of the SCN (5 Hz), or chemical excitation of SCN neurons by local application of glutamate, caused a sustained and reversible decrease of the amplitude of EPSCs evoked by electrical shocks (0.5 ms, 100 μA) delivered to the OVLT at 0.1 Hz. This observation suggests that a substance released by the axon terminals of SCN neurons can reduce the efficacy of neurotransmission at glutamatergic OVLT→SON synapses.

(Figure 3 near here)

Decreases in synaptic strength could be due to an SCN-mediated decrease in glutamate release from the axon terminals of OVLT neurons, or to a decrease in the postsynaptic responsiveness of MNCs to glutamate. As illustrated in figure 4(A,B), an analysis of the asynchronous EPSCs (aEPSCs) trailing the compound response evoked by OVLT stimulation

revealed that quantal amplitude is unaffected at modulated synapses during activation of SCN neurons, but that this procedure causes a significant decrease of aEPSC frequency. These findings indicate that activation of the SCN reduces the probability of glutamate release from the axon terminals of OVLT neurons while having no effect on the postsynaptic responsiveness of MNCs. Indeed, responses evoked in MNCs by local application of exogenous AMPA were not affected by SCN stimulation [70]. Additional observations were also consistent with this hypothesis. Specifically, the percentage of trials in which no response was observed when stimulating the OVLT at low intensity (i.e. the rate of "synaptic failures") was found to increase during SCN stimulation at 5 Hz, suggesting that the probability of transmitter release by the nerve endings of OVLT neurons is lowered by this procedure. Moreover, the separate responses mediated by AMPA and NMDA receptors at OVLT→SON synapses were inhibited by equivalent amounts during stimulation of the SCN (Figure 4C,D). Activity-dependent changes in postsynaptic strength associated with changes in AMPA receptor density commonly occur in the absence of concurrent changes in NMDA receptor density. Taken together, these observations indicate that the effects of SCN stimulation are mediated by a decrease in the probability of glutamate release at the presynaptic terminals of OVLT neurons that make synaptic contacts with MNCs.

(Figure 4 near here)

Presynaptic inhibition of neurotransmitter release is commonly mediated by a reduction in the efficacy of Ca^{2+} influx through the voltage gated calcium channels that are activated upon arrival of an action potential into a nerve ending [80]. Under such conditions, there is generally an increase in the relative facilitation of transmitter release caused by the arrival of a second impulse within a short interval (~40 ms) because the temporally summated intra-terminal Ca^{2+} concentration rises by a proportionally greater extent when calcium channels are inhibited than

when they are not [81]. Consequently, presynaptic inhibition of transmitter release mediated via reduced Ca^{2+} influx is normally associated with an increase in the paired pulse ratio (PPR) measured as EPSC2/EPSC1, where EPSCs 1 and 2 are the amplitudes of EPSCs evoked 40 ms apart. In agreement with this principle, presynaptic inhibition of OVLT→SON synapses caused by activation of $GABA_B$ receptors, which attenuate presynaptic Ca^{2+} influx, is accompanied by a significant increase in PPR at OVLT→SON synapses [82]. Similarly, presynaptic inhibition of glutamatergic synapses formed on MNCs induced by the activation of adenosine A1 receptors [83] and metabotropic glutamate receptors [84] is also associated with an increase in PPR.

However in these experiments we found that the inhibition of OVLT→SON synapses caused by activation of the SCN was not accompanied by any change in PPR (Figure 4E,F). This observation suggests that the mechanism by which SCN neurons inhibit glutamate release at these synapses does not involve a graded reduction in voltage-gated calcium influx, but rather an all-or-nothing suppression of calcium-mediated transmitter release at a subset of the synapses. In this form of inhibition, termed *presynaptic silencing* [85-87], a proportion of the nerve endings simply stop releasing glutamate and the axon terminals that remain functional retain a normal release probability. As illustrated in figure 5, we therefore hypothesize that the number of functional OVLT→SON synapses that are available for mediating basal and osmotically-driven excitatory drive information transfer varies according to the time of day. When clock neurons are highly active (e.g. during the MSP), the fraction of OVLT→SON synapses that are functionally active is low due to activity-dependent presynaptic silencing. When clock activity is low (e.g. during the LSP), a greater proportion of the synapses become active and can better relay the osmosensory signals provided by OVLT neurons.

(Figure 5 near here)

VP is a mediator of the clock's inhibitory effect.

Previous studies have shown that a large number of neurotransmitters and peptides are expressed in various subsets of SCN neurons [64]. Moreover, it remains possible that the final effector responsible for the presynaptic modulation of OVLT→SON synapses is released not by the axon terminals of the SCN neurons, but by a local relay neuron. Determining the nature of the neuromodulator signal is therefore a difficult task. Preliminary experiments using a pharmacological approach were performed to explore the possible involvement of transmitters that have already been shown to promote presynaptic inhibition of excitatory synapses in the SON and which are known to be present in the SCN. The 5 candidate receptors tested so far were adenosine A_1 receptors [83], $GABA_A$ receptors (excluded by the presence of bicuculline), $GABA_B$ receptors [82, 88], metabotropic glutamate receptors [84, 89], and V1a VP receptors [90]).

As summarized in Figure 6, the inhibitory effect of SCN stimulation was only significantly antagonized by 2 μM [d(CH(2))(5)(1)-O-Me-Tyr(2)-Arg(8)]-vasopressin (MC, Manning compound), an antagonist of the VP V1a and oxytocin receptors [91]. Although a high proportion of SCN neurons express VP [56, 64, 66, 92], previous work has shown that excitatory synaptic transmission in the SON can also be inhibited by activity-dependent release of VP [93, 94] or OT [88, 94] from the dendrites of MNCs. Since $GABA_A$ receptors were blocked in our experiments, electrical stimulation of the SCN typically evoked unopposed EPSCs in MNCs. We therefore considered the possibility that repetitively stimulating the SCN input might be mediating its effect by causing an excitation of the surrounding MNCs and that dendritic release of VP or OT from those neurons would then inhibit excitatory afferents to the cell being recorded via a paracrine effect. However repetitively stimulating the OVLT pathway at 20 Hz had no effect on EPSCs evoked by low frequency stimulation of the SCN, whereas repetitive stimulation of the SCN significantly inhibited the OVLT→SON synapses when using the same stimulation

electrodes [70]. Since the magnitudes of EPSCs evoked by both electrodes were comparable, the degree of postsynaptic excitation evoked in neighboring cells during repetitive stimulation of the OVLT or SCN should have been comparable. This finding therefore implies that the source of VP or OT mediating presynaptic inhibition is not the surrounding MNCs. Because SCN neurons do not express OT [95] and VP is not expressed in other types of neurons in the vicinity of the SON, the result suggests that VP released by the axon terminals of SCN neurons may be responsible for part of the clock's effect on OVLT afferents.

(Figure 6 near here)

Concluding Remarks.

At the level of the whole organism circadian rhythms often manifest themselves as overt changes in behaviour dictated by a phase-locking of the sleeping and active periods to the local solar cycle (e.g. wheel running, feeding behavior). These rhythms nonetheless reflect the influence of the master clock since they persist in the absence of light cues and can be abolished by SCN lesions. Recent work has shown that individual organs and tissues also display a wealth of less conspicuous rhythms in gene or protein function that presumably adapt cellular biochemistry and function to changing physiological demands during the circadian cycle. Although many of these rhythms may involve local molecular clocks [53, 64, 65], it is generally believed that the SCN still plays a role in maintaining the proper timing of these peripheral clocks. How the SCN orchestrates all of the body's rhythms remains a mystery and this control may well involve both neural efferents and humoral signals.

Previous work has shown that VP neurons in the SON are directly inhibited via a GABAergic projection from the SCN [71]. While it is clear that an activity-dependent inhibition

of MNCs mediated by GABAergic synapses might contribute to the circadian control of circulating VP concentration, it is not clear how such a direct synaptic effect could alter the responsiveness of MNCs to osmoreceptor inputs. The work summarized here indicates that SCN clock neurons can modulate information transfer at excitatory OVLT→SON synapses in a manner that is consistent with rising VP levels and increased osmoresponsiveness during the LSP. Additional work is needed to clarify the role of the direct inhibitory output of the SCN to VP neurons, and to identify other mechanisms by which the SCN may participate in the circadian control of VP secretion.

In addition to the many unanswered questions that remain regarding clock output and function, four specific issues regarding the circadian control of VP release should now be addressed. First, previous studies *in vivo* [96] have shown that sEPSPs are required to sustain the phasic pattern of firing adopted by VP neurons to maximize neurosecretion [11]. It is therefore tempting to speculate that the elevated firing rate of SCN neurons during the MSP might reduce the percentage of VP neurons displaying phasic firing through a reduction of sEPSP amplitude, and that reversal of this effect during the LSP might contribute to the enhancement of VP secretion during this period. Second, the activity of clock neurons declines during the MSP to LSP transition and this coincides with a progressive facilitation of osmoregulated MNC firing and systemic VP release [46]. Although this is consistent with an activity-dependent inhibition of OVLT→SON synapses [70], the full circadian electrical activity of clock neurons is not exactly 180 degrees out of phase with that of VP release, suggesting that other mechanisms mediate clock output or that other factors supersede it's influence during other parts of the circadian cycle. Third, the identity of the modulators and molecular mechanisms involved in mediating presynaptic silencing remain to be fully established, as is the possible involvement of this mechanism in other central circuits. Fourth, the involvement of clock mediated activity-

dependent presynaptic inhibition of osmosensory afferents is consistent with the circadian pattern of VP release in nocturnal rodents. However in diurnal animals (e.g. humans) the VP surge that occurs during the LSP is 180 degrees out of phase with the light cycle. This suggests either that light-related activity is reversed in clock neurons innervating the SON in such animals, or that entirely different mechanisms are at play in different species. Additional studies are required to address these important issues.

Acknowledgements.

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Figure Legends.

Figure 1

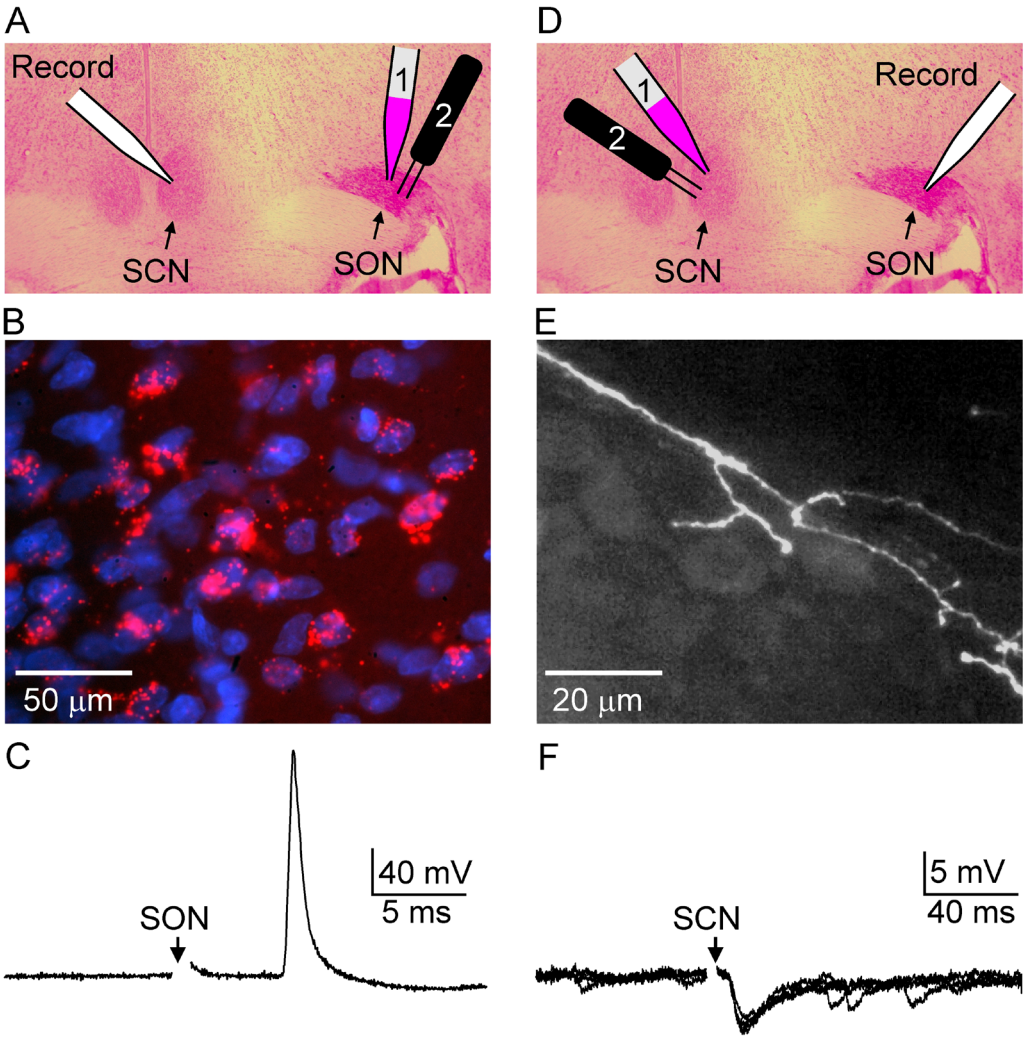

Figure 1. SCN neurons project to SON. A, illustration of retrograde analysis techniques. B, Photomicrograph of histological section stained with DAPI to indicate nuclei (blue) taken through the SCN of a rat several days after fluorescently labelled microspheres (red) were injected into the SON *in vivo* (#1 in A). Note that many SCN neurons have transported the microspheres. C, intracellular recording of membrane voltage shows an SCN neuron recorded in a horizontal rat brain slice that can be antidromically activated by an electrical stimulus applied to the SON (#2 in A). D, Schematic illustrating orthograde approaches. E, Photomicrograph showing a small part of the SON in a paraformaldehyde-fixed rat brain slice where DiI had been placed into the SCN several days before (#1 in D). Note the labelled branched axon that features terminations in close apposition with SON neurons. F, three superimposed sweeps show intracellular voltage in an SON neuron recorded in a horizontal rat brain slice. Note that electrical stimulation of the SCN (#2 in D) evokes a prominent IPSP. Panels B & D reproduced and adapted from [70].

Figure 2

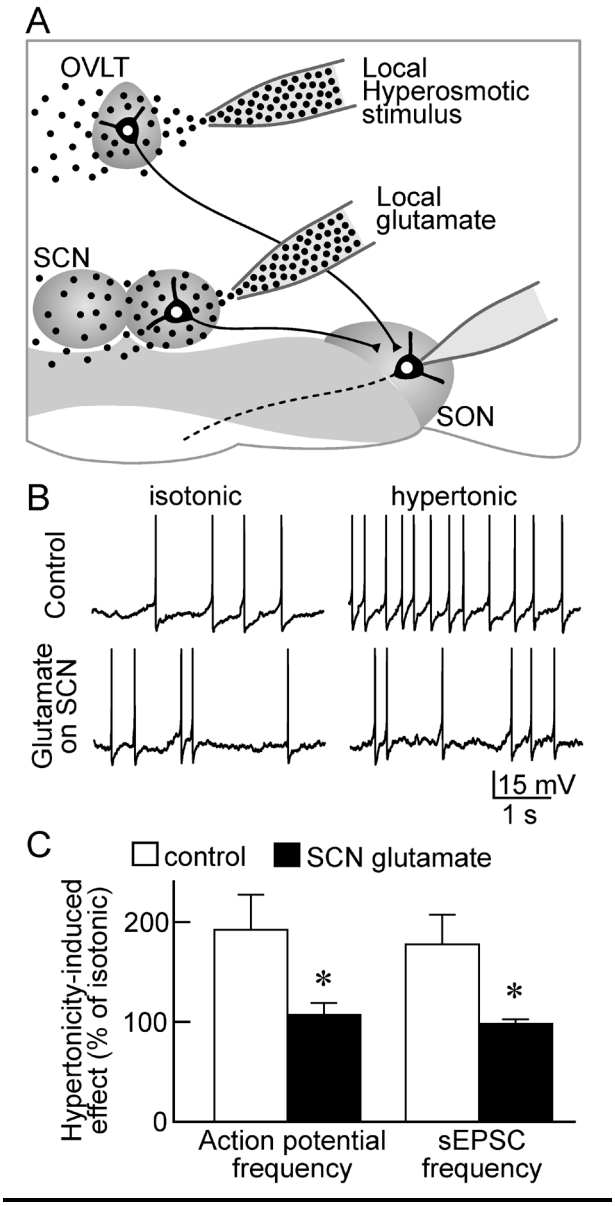

Figure 2. Activation of the SCN inhibits osmotic activation of SON neurons via the OVLT→SON pathway. A, schematic diagram illustrates the experimental approach. B, membrane voltage traces taken from an SON neuron show that action potential firing rate is increased by hyperosmotic stimulation of the OVLT under control conditions, but not when glutamate is locally applied to the SCN. C, bar graphs quantify the hypertonicity-induced effects, expressed as percent of baseline activity, on action potential and sEPSC frequency in control conditions and when SCN neurons are excited by local application of glutamate. Note the significantly reduced responses when the SCN is activated. All panels adapted from [70].

Figure 3

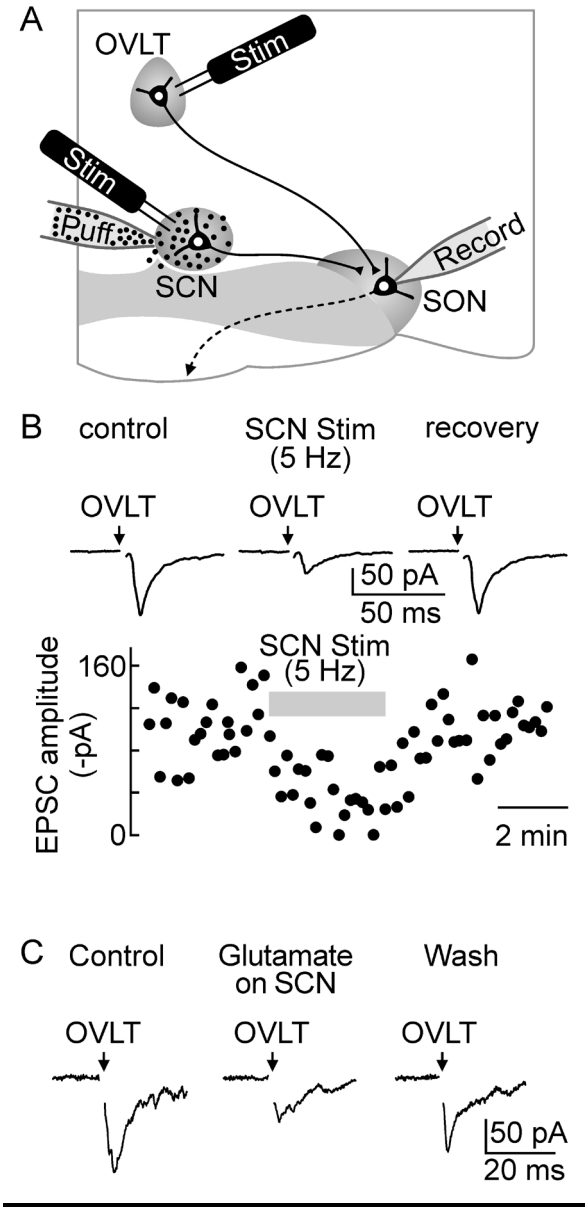

Figure 3. Activation of the SCN inhibits glutamatergic OVLT→SON synapses. A, schematic diagram illustrates the experimental approach. Bicuculline was present throughout to block $GABA_A$ receptors. B, Top traces show EPSCs evoked in an SON neuron by electrical stimulation of the OVLT (arrows) before (control), during SCN stimulation at 5 Hz (SCN Stim), and after recovery. The graph shown below plots the amplitude of each individual EPSC evoked in this experiment. Note that SCN stimulation reduces the amplitude of the EPSCs. C, Traces show examples of EPSCs recorded from another SON neuron under control conditions, when glutamate is applied over the SCN, and after wash. All panels adapted from [70].

Figure 4

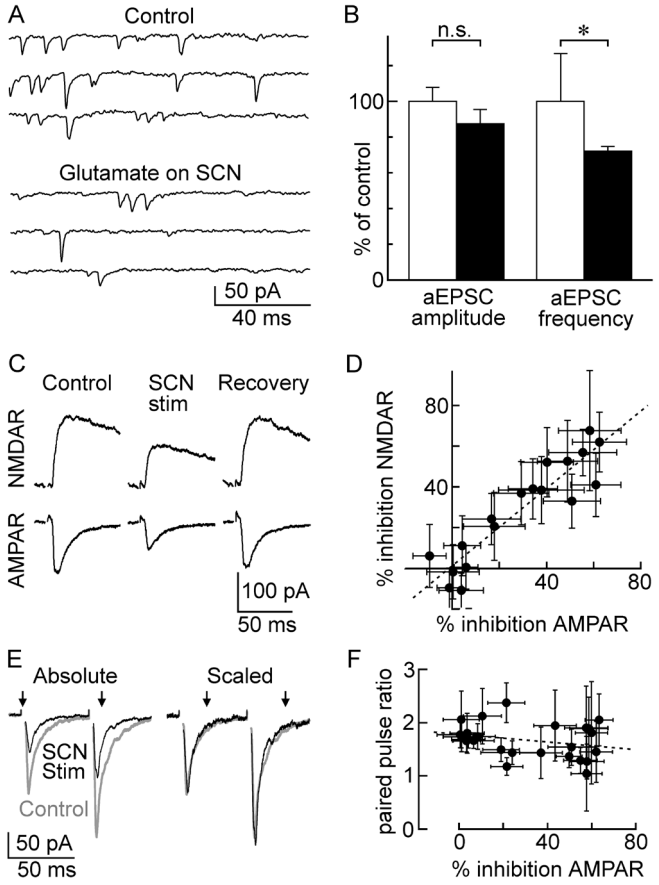

Figure 4. Activation of the SCN causes presynaptic silencing at OVLT→SON synapses. Traces show the small aEPSCs that follow the larger EPSC (not shown) evoked in an SON neuron by stimulation of the OVLT under control conditions and while glutamate is being applied to the SCN. Each trace is a separate sweep. B, bar graphs quantify data obtained in many SON neurons from traces such as those shown in A. Note that the frequency of aEPSCs is significantly reduced by application of glutamate to the SCN (black bars) relative to control (white bars). C, examples of synaptic responses evoked in an SON neuron by stimulation of the OVLT before, during and after stimulation of the SCN at 5 Hz (SCN Stim). Top traces were recorded at +40 mV, where the late component reflects mainly current flowing through NMDA receptors (NMDAR). Lower traces show EPSCs recorded at -60 mV, reflecting mainly currents flowing through AMPA receptors (AMPAR). D, graph plots percent inhibition of the synaptic NMDAR current as a function of AMPAR inhibition during the onset and recovery phases of the SCN-induced effect. Note the correlated inhibition of both receptors. E, Pairs of EPSCs evoked in an SON neuron before (grey, control) and during SCN stim at 5Hz. Traces at right show the same traces scaled to normalize the amplitude of the first EPSC. F, Plot shows mean values of paired pulse ratio (EPSC2/EPSC1) expressed as a function of the mean SCN-mediated inhibition of the AMPAR observed during the onset and recovery phases of the SCN-induced effect. Note the lack of change in PPR. All panels adapted from reference [70].

Figure 5

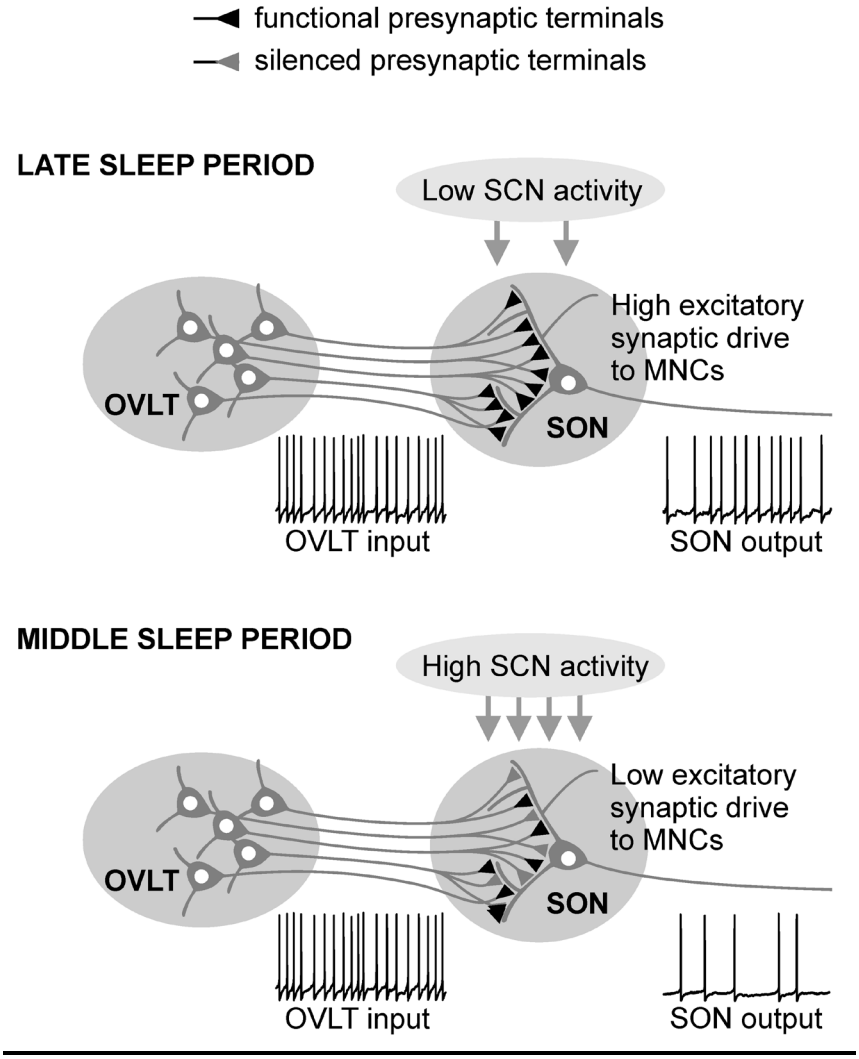

Figure 5. SCN output modulates the proportion of functional OVLT→SON synapses. Schematic diagram illustrating the effects of clock (SCN) activity on the proportion of OVLT→SON presynaptic terminals that are functionally active (capable of releasing glutamate). During the middle sleep period, when SCN activity is high, the proportion of functional presynaptic terminals is reduced, which reduces the ability of OVLT "input" signals to drive "output" activity from SON neurons. During the LSP clock activity is reduced, which disinhibits the nerve terminals and increases the efficacy of the OVLT→SON pathway.

Figure 6

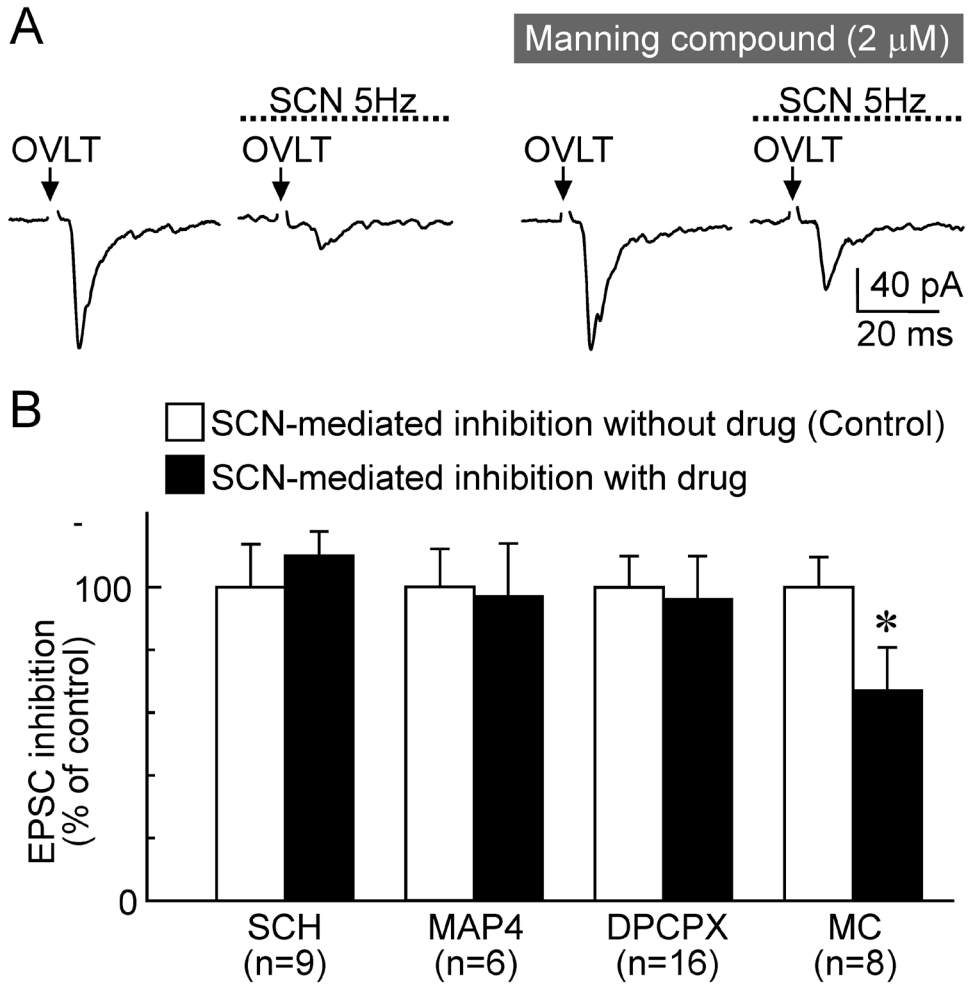

Figure 6. Vasopressin mediates part of the SCN effect on OVLT→SON synapses. A, examples of EPSCs evoked in an SON neuron by electrical stimulation of the OVLT (arrows) before and during SCN stimulation at 5 Hz (dotted line). The pair of traces on the left were obtained in the absence of a vasopressin receptor antagonist. The pair on the right (grey bar) was obtained in the presence of Manning Compound (MC, a V1A receptor antagonist). Note that the degree of inhibition caused by SCN stimulation is reduced in the presence of MC. B, bar graphs plot the mean (±s.e.m.) effects of various receptor antagonists on the degree of SCN-mediated inhibition in groups of cells (numbers of cells shown in brackets), expressed as percent of the effect observed without drug. SCH is a $GABA_B$ antagonist, MAP4 is a metabotropic glutamate receptor antagonist, DPCPX is an A1 adenosine receptor antagonist. Panels adapted from reference [70].

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