Membrane Properties and Morphology of Vasopressin Neurons in Slices of Rat Suprachiasmatic Nucleus

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Pennarts, C.M.A., N.P.A. Bos, M.T.G. De Jeu, A.M.S. Geurtsen, M. Mirmiran, A. A. Sluiter, and R. M. Buijs. Membrane properties and morphology of vasopressin neurons in slices of rat suprachiasmatic nucleus. *J. Neurophysiol.* 80: 2710–2717, 1998. Vasopressin (VP) neurons in the suprachiasmatic nucleus (SCN) are thought to be closely linked to neural mechanisms for circadian timekeeping. To gain insight into the cellular–physiological principles that govern spike-driven VP release and to examine whether VP cells can be electrophysiologically and morphologically identified by a unique combination of features, we recorded membrane properties by whole cell patch-clamp methods and stained the cells with biocytin. In current-clamp mode, VP neurons recorded during subjective daytime expressed a clear time-dependent inward rectification but no pronounced low-threshold Ca^{2+} potential after hyperpolarizing current pulses. Their spontaneous firing rate varied between 0.6 and 13.4 Hz and was generally tonic and irregular. Spike afterhyperpolarizations (AHPs) were steeply rising and monophasic. Spikes were preceded by depolarizing ramps mediated by a slow component of Na^{+} current. Spike trains evoked by depolarizing current pulses displayed frequency adaptation and were usually followed by an AHP lasting 0.5–2.0 s. Spontaneous postsynaptic potentials were present in a majority of cells. Voltage-clamp recordings revealed a Ba^{2+} -sensitive K^{+} current that exerts a tonic, hyperpolarizing influence on the membrane potential. This set of membrane properties was not significantly different from other cells in the dorsomedial region and is characteristic for cluster I cells, which were described previously and are widely encountered throughout the SCN. None of the cells could be classified as belonging to cluster II or III, which were indeed found mainly outside the dorsomedial region. Morphologically, single VP neurons were characterized by compact, mono- or bipolar dendritic branching patterns and numerous varicosities throughout the dendrites. They generally possessed few axon collaterals, most of which remained inside the boundaries of the SCN but were occasionally seen to project to SCN target areas. In conclusion, VP neurons in the SCN express several active membrane properties, including time-dependent inward rectification, frequency adaptation in spike trains, monophasic spike AHPs, and Ba^{2+} -sensitive K^{+} current. VP release is proposed to be governed by tonic and irregular patterns of spontaneous firing. The electrophysiological and cytological properties of VP neurons are representative for a majority of SCN cells and define them as a subset of previously defined cluster I cells.

INTRODUCTION

The dorsomedial neuropil of the suprachiasmatic nucleus (SCN), a hypothalamic region that harbors the pacemaker

of circadian rhythms, contains a high-density of vasopressin (VP)-synthesizing neurons (Castel et al. 1990; Swaab et al. 1975; Van den Pol and Tsujimoto 1985; VandeSande et al. 1975). These neurons are intimately linked to the biological clock because VP release exhibits a clear diurnal rhythm both in the cerebrospinal fluid (Schwartz and Reppert 1985) and in perfusates from SCN slices and cultures (Earnest and Sladek 1986; Shinohara et al. 1995). VP peptide content and messenger ribonucleic acid display a circadian rhythm as well (Uhl and Reppert 1986), even under free-running conditions (Cagampang et al. 1994). VP neurons in SCN were implicated in the transfer of circadian output to target areas, regulating physiological functions such as corticosterone release (Kalsbeek and Buijs 1996). They may also play a role in regulating photoperiodic adaptation (Hofman et al. 1993). Despite the indications for a role of VP neurons in relaying clock output to target structures, this peptide is unlikely to play an exclusive role in the expression of circadian rhythmicity. First, it was observed that the VP-deficient Brattleboro rat retains circadian rhythmicity in a number of physiological functions (Peterson et al. 1980), and SCN slices prepared from these rats display a circadian rhythm in spontaneous firing rate (SFR) (Ingram et al. 1996). Second, in SCN cultures treated with antimitotics, Shinohara et al. (1995) observed nonsynchronous rhythms in release of VP and vasoactive intestinal polypeptide (VIP). These and other findings raise the possibility that the SCN contains a redundant timekeeping system composed of multiple circadian oscillators.

Besides projecting to target structures outside SCN, VP neurons form a dense network of local circuits both within and outside the dorsomedial area. They receive inputs from other SCN neurons such as the more ventrally located VIP neurons (Ibata et al. 1993) and innervate hetero- and homologous neural elements within the SCN (Castel et al. 1990; Daikoku et al. 1992; Van den Pol and Gorcs 1986). Concerning the electrophysiological action of VP on target cells, an excitatory, V_1 receptor-mediated action of exogenous VP was reported (Ingram et al. 1996; Liou and Albers 1989). Because VP colocalizes with γ -aminobutyric acid (GABA) in SCN (Buijs et al. 1995), these cells may also generate inhibitory output and may thus employ a dual mode of control on the excitability of their postsynaptic targets.

The aim of this study was to identify the membrane properties and morphology of single VP neurons by whole cell recordings in acutely prepared slices of rat hypothalamus.

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This identification may further our understanding of the functions of VP in the organization of the biological clock in various ways. First, it may shed light on the conversion of synaptic input into dynamically organized spike trains that trigger VP release at axon terminals and varicosities. Second, it allows to assess whether a specific peptidergic phenotype of SCN cells expresses a unique electrophysiological "fingerprint" by which it can be identified while making recordings. Related to this issue is the recent description of three cell clusters in SCN distinguished by electrophysiological criteria (Pennartz et al. 1998). From this partitioning the question logically follows whether the subpopulation of VP cells is congruent with one of these cell clusters. Third, it provides a basis for investigating whether the circadian rhythm in VP release is caused by an underlying rhythm in SFR of VP cells or by rhythmic processes operating at the level of release sites. In general, this combined immunocytochemical and electrophysiological study may supplement our relatively advanced neurochemical and anatomic knowledge of VP cells with insights into their electrophysiological behavior.

METHODS

Slice preparation and whole cell recording

Brain slices containing the SCN were obtained from male Wistar rats (180–300 g) after procedures described previously (Pennartz et al. 1997, 1998). In brief, rat brains were excised from the skull after decapitation and placed in ice-cold artificial cerebrospinal fluid (ACSF). A block of tissue containing the hypothalamus was dissected, and transverse slices 200 μ m thick were cut on a vibratome (Vibroslicer, Campden, UK). Before recording, the slices were stored in a chamber gassed with 95% O₂–5% CO₂ and kept at room temperature (22–25°C) for $\geq$ 45 min. After transfer to the recording chamber, slices were superfused at a pump speed of 1.5–2.5 ml/min with oxygenated ACSF (composition in mM: 124.0 NaCl, 3.0 KCl, 26.2 NaHCO₃, 1.0 NaH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, and 11.0 D-glucose, pH 7.3, temperature 22–25°C unless otherwise noted). Patch clamping under visual control was performed with an upright microscope (Zeiss Axioskop) equipped with a $\times$ 40 Hoffman modulation contrast objective (cf. Stuart et al. 1993). Whole cell recordings were made with pipettes filled with (in mM) 136.0 K⁺ gluconate, 10.0 KCl, 10.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.5 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5.0 biocytin, 2.0 Na₂ATP (pH 7.3, 270–275 mosmol, pipette resistance 4–8 M Ω). After establishing a gigaseal between pipette and membrane $>$ 3 G Ω , the membrane was ruptured by mouth suction, allowing biocytin to diffuse into the neuron. Recordings were made in current-clamp mode unless mentioned otherwise. Voltage or current traces were recorded by an Axopatch 1D or Axopatch 200 amplifier and acquired with the use of pClamp 6.02 and Axotape 2.0 (all from Axon Instruments). Sampling rates were 5 or 10 kHz. All membrane potential values were corrected for the liquid junction potential (–13 mV according to the method described by Neher 1992). Membrane properties were quantified as described by Pennartz et al. (1998). All recordings were made in the subjective day phase, between circadian time 7 and 11. Groups of cells were statistically compared with the use of Student's *t*-test for unpaired samples and Mann-Whitney's *U* test.

Biocytin labeling and three-dimensional reconstruction

During an experiment, the positions of all recorded neurons were marked in an overview sketch of the slice to facilitate identification

TABLE 1. *Membrane properties of vasopressin neurons in suprachiasmatic nucleus*

Parameter	Mean
Resting membrane potential, mV	-53.5 ± 1.1
Input resistance, G Ω	1.45 ± 0.12
Time constant, ms	29.4 ± 2.3
Action potential amplitude, mV	68.1 ± 2.0
Action potential width, ms	2.2 ± 0.1
Spontaneous firing rate, Hz	3.7 ± 0.8
Coefficient of variation for spike intervals	0.46 ± 0.06

Values are means $\pm$ SE. For methods of quantification, see text. These data are based on 17 cells recorded at room temperature.

of labeled neurons after the immunocytochemical procedure. Slices containing biocytin-filled neurons were fixed for $\geq$ 12 h in 4% paraformaldehyde or 4% formaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4–7.6) at 4°C and stored for at least 24 h in Tris-buffered saline (TBS; pH 7.4–7.6; 0.05 M Tris and 0.15 M NaCl) with 0.05% sodium azide. After washout of sodium azide in TBS for 1 h, slices were incubated overnight at 4°C with rabbit-anti-rat neurophysin (A. G. Robinson, University of Pittsburgh, PA) diluted 1:2000 in a mix of TBS, 0.25% gelatin, and 0.5% Triton. Subsequently, slices were washed in TBS and subsequently incubated with 1) donkey-anti-rabbit-Cy5 or donkey-anti-rabbit-FITC (1:400) and 2) streptavidin-Cy3 (1:1000; Jackson Immuno-research) (Romijn et al. 1996), diluted in a mix of TBS, 0.25% gelatin, and 0.5% Triton at pH 7.6. Slices (200 μ m) were mounted on coated glass slides and covered with Vectashield (Vector Labs). Because SCN cells do not express oxytocin (Swaab et al. 1975; Van den Pol and Tsujimoto 1985; Van den Sande et al. 1975), it can be safely assumed that the VP and oxytocin precursor peptide neurophysin specifically labeled VP cells. A confocal scanning laser microscope (Zeiss-Kontron) was used to produce optical sections (1 μ m) of fluorescently labeled cells, emitting light at 543 and 633 nm to excite Cy3 and Cy5, respectively. These sections were superimposed to reconstruct cell morphology in three dimensions. Cells were labeled "VP positive" if their somata were clearly delineated and were clearly more fluorescent than the background. A cell was only classified as "VP negative" if a sufficient number of positively stained cells was identified in the same histological section as containing the cell of interest.

RESULTS

Basic membrane properties and time-dependent rectification

On a total of 91 biocytin-labeled cells that were immunostained for neurophysin, 24 neurons were identified as VP positive and were acceptable by electrophysiological standards (spike amplitude of at least 55 mV as measured from baseline). Seventeen neurons were recorded at room temperature, and 7 cells were recorded at 30–31°C. The quantitative data given below pertain to the former group and are summarized in Table 1. The latter group generally displayed quantitatively similar properties, although the spike width at half-maximal amplitude was shorter (1.8 ± 0.2 ms; mean $\pm$ SE) than at room temperature (2.2 ± 0.1 ms; not significant). All VP-positive cells were located in the dorsomedial SCN.

The membrane potential of VP cells was -53.5 ± 1.1 mV ($n = 17$). Injection of square, hyperpolarizing current pulses was employed to evaluate the input resistance, time constant, and presence of hyperpolarization-activated con-

FIG. 1. Membrane properties of vasopressin (VP) cells in suprachiasmatic nucleus (SCN) recorded in current-clamp mode. *A*: hyperpolarizing current pulse (-52 pA; indicated below trace) revealed time-dependent inward rectification (activation of H-current; indicated by asterisk) in this cell. After pulse termination, a few rebound spikes were observed, whereas a large rebound depolarization was absent. Note depolarizing ramp preceding spikes (example indicated by arrow). *B*: spike average from the same cell illustrating its monophasic spike afterhyperpolarization (AHP); spikes were taken from a record part of which is shown in *C*. *C*: spontaneous firing pattern was characterized by irregular firing and a ragged appearance of the membrane potential in between spikes. *D*: depolarizing current pulse ($+27$ pA) evoked a spike train showing frequency adaptation. The spike train also exhibited a mild, progressive decrease in spike amplitude and was followed by a slow train AHP. The dotted line is an extension of the baseline. All records in this figure were taken from the same cell. Calibration: vertical bar 30 mV (*A–D*); horizontal bar (*A*) 500 ms; (*B*) 50 ms; (*C*) $1,200$ ms; and (*D*) 300 ms.

ductances. The input resistance and time constant, estimated by monoexponential fits to hyperpolarizing voltage responses well below spike threshold, amounted to 1.45 ± 0.12 G Ω and 29.4 ± 2.3 ms, respectively. From the 17 VP cells, 15 (88%) displayed time-dependent rectification when sufficiently hyperpolarizing current pulses were injected (Fig. 1*A*). This “depolarizing sag” has been shown to reflect activation of the H-current (Akasu et al. 1993; De Jeu and Pennartz 1997), and indeed the characteristic slow activation of this current by hyperpolarization was revealed in five of six neurons (83%) held under voltage clamp (Fig. 2, *A* and *B*). Some SCN neurons were also described to generate a marked “rebound depolarization” after termination of the hyperpolarizing current pulse. This phenomenon can be ascribed to activation of a low-threshold Ca^{2+} potential (Akasu et al. 1993; Huguenard 1996), although continued activation of the H-current may also contribute. None of the VP cells generated a large rebound depolarization, although the spike density was usually enhanced on pulse termination (Fig. 1*A*). Membrane hyperpolarization to levels around -90 mV allowed to assess the presence of spontaneous postsynaptic potentials (PSPs), which in the SCN are primarily of an inhibitory nature and are mediated by GABA_A receptors (Jiang et al. 1995; Kim and Dudek 1992; unpublished observations of authors). These spontaneous PSPs were present in 59% of the VP cells, suggesting that this population receives pronounced GABAergic input.

Spontaneous firing behavior

A large majority of VP cells (16/17; 94%) fired spontaneously; the spontaneous firing rate (SFR) ranged between 0.6 and 13.4 Hz and was 3.7 ± 0.8 Hz on average. Action potentials were quantitatively assessed from spontaneous firing patterns generated in the first 10–15 min of recording. This restricted time window was used because a rundown in spike amplitude, possibly caused by whole cell dialysis, became gradually manifest in a majority of cells. The rising and falling slopes of the action potentials in VP cells were monophasic. The action potential amplitude (measured with

respect to baseline) and spike width at half-amplitude were 68.1 ± 2.0 mV and 2.2 ± 0.1 ms, respectively. Spikes were invariably preceded by spontaneous depolarizing ramps having slope values of 0.05–0.50 mV/ms. These ramps or prepotentials have been shown to be mediated by a slow component of Na^+ current (Pennartz et al. 1997). Previously, we described a considerable heterogeneity in the shape of the spike afterhyperpolarization (AHP), which may vary from steeply rising and monophasic to slowly rising and biphasic (Pennartz et al. 1998). This heterogeneity was not encountered in VP cells as they uniformly displayed steeply rising, monophasic AHPs (Fig. 1*B*).

VP cells typically generated irregular patterns of spontaneous firing (Fig. 1*C*). Spike intervals were characterized by a ragged appearance; this may be due to spontaneous PSPs and to subthreshold Na^+ channel activity (cf. Pennartz et al. 1997). As a measure of irregularity in firing, we quantified the coefficient of variation (CV) of spike intervals, which equals the SD divided by the mean interval (Groos and Hendriks 1979). Although the high value of the mean CV (0.46 ± 0.06 ; $n = 16$) confirms the irregularity of these cells, it is important to take into account the dependence of the CV on the SFR. Previously we have shown that cells firing at rates below ~ 1.5 Hz have a strong tendency to fire irregularly, and they tend to fire regularly at rates above 5.0 Hz (Pennartz et al. 1998; see also Kim and Dudek 1993). When we restricted the analysis to VP cells with $1.5 \leq \text{SFR} \leq 5.0$ Hz, the mean CV was high as well (0.52 ± 0.07 ; $n = 9$). Thus irregular firing appears to be a constant feature of VP cells even at intermediate levels of SFR. None of the VP cells exhibited bursting behavior.

Properties of high-frequency spike trains

The spike frequency was temporarily boosted to higher levels by injecting positive current pulses into the cell. Although the cells included in this study generated single spikes of considerable amplitude, some of them generated spike trains with rapidly decreasing spike amplitude, a phenomenon that may be caused by whole cell dialysis. When

FIG. 2. H-current and Ba²⁺ sensitive K⁺ current in a VP cell held under voltage-clamp. *A*: control traces of current responses to voltage step commands. Note the slowly increasing, inward current (I_H) evoked by strongly hyperpolarizing voltage steps. $\circ$ and $\bullet$: time at which the instantaneous current and steady-state current were measured, respectively. The voltage-clamp commands are plotted in the bottom panel of *B* and consisted of a series of steps beginning at -120 mV and going up to -10 mV with an increment of 10 mV. Holding potential was -50 mV. The maximal error in voltage clamping, as estimated by the formula $V_{err} = R_s I$ (Armstrong and Gilly 1992) (R_s is the series resistance and V_{err} the voltage-clamp error) amounted to 0.7 mV in this cell. Series resistance was compensated by 80%. The experiment was performed in the presence of tetrodotoxin (1 μ M). *B*: on Ba²⁺ application (500 μ M), both the inward and outward instantaneous currents were reduced, whereas the H-current remained largely unaffected. *C*: current-voltage plot illustrating the voltage dependence of the instantaneous (I_{inst}) and steady-state currents (I_{ss}) under control conditions (from data in *A*). The difference between the $\circ$ and $\bullet$ indicates the magnitude of the H-current. *D*: current-voltage plot of the Ba²⁺-sensitive K⁺ current. This plot was obtained by subtracting steady-state values of current measured in Ba²⁺ conditions from control values. The reversal potential lies at -70 mV. Calibration: 40 pA, 55 mV and 500 ms for *A* and *B*.

the analysis was restricted to cells showing well-maintained spike trains ($n = 11$), a moderate frequency adaptation was usually found on injection of 10- to 30-pA pulses (Fig. 1*D*). Maximal frequencies attained just after spike train onset ranged from 26 to 50 Hz (mean 34.5 ± 2.4 Hz). After terminating the current pulse, a clear spike train AHP, 0.5–2.0 s in duration, was found in 82% of the VP cells (Fig. 1*D*). This AHP occurred in conjunction with moderate to strong frequency adaptation and is most likely due to activation of a Ca²⁺-dependent K⁺ current (presumably carried by SK_{Ca} channels) (McLarnon 1995; Sah 1996; Storm 1990).

Barium-sensitive K⁺ conductance

Although it was generally difficult to identify ionic currents in VP cells pharmacologically because of their fragility, we investigated a particular nonligand gated K⁺ conductance in three VP cells. This conductance was recently characterized as a Ba²⁺-sensitive, weakly outward rectifying K⁺ conductance (De Jeu, Geurtsen, and Pennartz, unpublished observations). This conductance directly regulates the membrane potential and thereby indirectly affects the SFR of SCN neurons. The three VP neurons were held under voltage clamp, and their inward and outward currents were investi-

gated by step protocols (Fig. 2). Under control conditions, cells generated both an instantaneous and a slowly increasing inward current in response to hyperpolarizing steps (Fig. 2, *A* and *C*). Under Ba²⁺ conditions (500 μ M) conditions, the slow (H-)current remained largely intact while the instantaneous component was consistently seen to decrease in amplitude (Fig. 2, *A* and *B*). Depolarizing voltage steps activated outward currents that were also reduced under Ba²⁺ conditions (Fig. 2, *A* and *B*). When the current traces from the control and barium condition were subtracted (Fig. 2*D*), a weakly outward rectifying I/V characteristic was obtained, in agreement with previous findings on SCN neurons in general (De Jeu et al., unpublished observations).

Morphology of VP cells

VP cells might lose a considerable amount of their peptide content during whole cell dialysis. We estimated the likelihood of identifying false-negative cells as a consequence of this potential problem by plotting the probability of recording a neurophysin-positive cell as a function of recording duration (from break-in to pipette withdrawal; most recordings lasted <10 min). As we did not identify any negative correlation between these parameters in this time

FIG. 3. Morphology of single VP neurons in SCN. *A*: superimposed optical sections, produced by a confocal scanning laser microscope (CSLM), of a neurophysin staining (represented in green) and a biocytin staining (represented in red) of the same slice. Three cells show clear overlap in neurophysin and biocytin staining (in yellow), whereas a 4th cell (in red) is neurophysin-negative. *B*: 3-dimensional reconstruction of a VP cell with 2 short processes and a characteristic varicose dendrite. *C*: 3-dimensional reconstruction of a VP cell with at least 3 axon collaterals (indicated by asterisks) coursing through SCN neuropil. Two primary dendrites are indicated by arrows. Both dendrites merge into much thinner axon collaterals; such configurations were previously referred to as "dendroaxons" (Pennartz et al. 1998). Because of the extremely fine caliber of the axons, some discontinuities in the CSLM composition are present. No collaterals of this cell were seen to cross the borders of the SCN. Scale bar: 10 μm for *A*, 30 μm for *B*, and 40 μm for *C*.

domain ($r = 0.23$; not significant), it seems reasonable to argue that short-lasting whole cell recordings can be used to reliably identify this peptidergic phenotype.

After biocytin staining and double labeling for neurophysin (Fig. 3*A*), the morphology of single VP cells ($n = 24$) was reconstructed with a confocal scanning laser microscope. Their dendritic branching patterns usually varied from monopolar to bipolar. Dendritic branching into secondary and tertiary dendrites was sparse. A majority of dendrites was varicose in appearance (Fig. 3*B*). The shape of the somata varied among round, ovoid, or conical, and polygonal shapes were rarely found. The minimal and maximal somatic diameters ranged from 7.0 to 18.7 and 9.2 to 22.7 μm , respectively. Spines on dendrites or somata were absent except in eight cases (33%). Axons originated from the soma or proximal dendrites and generally branched into a few local collaterals (range 0–9; mean 2.1 ± 0.5 ; Fig. 3*C*). A few VP cells could be identified as projection neurons as their axons coursed toward and into target structures unilateral to the SCN, i.e., the paraventricular nucleus of the hypothalamus, the subparaventricular zone, medial preoptic area, or into the contralateral SCN. These projection neurons also possessed multiple axon collaterals that stayed within the boundaries of the SCN. The percentage of VP projection neurons may well be higher than suggested here because axons running outside the plane of the slice were lost during the cutting procedure.

Comparison with other cell groups in the SCN

The membrane properties and morphology of VP cells were compared with other cell groups in the SCN in two ways. First, we sought to distinguish VP-positive from VP-negative cells located in the dorsomedial SCN. This comparison provides information especially about cellular heteroge-

neity of peptidergic phenotypes in this specific SCN subregion. Non-VP and VP cells in the dorsomedial SCN were highly similar in their electrophysiological and morphological properties. The properties listed in Table 1 were not significantly different in these two groups of cells, although action potentials in VP cells tended to be smaller in amplitude ($P < 0.10$).

We previously described a partitioning of SCN neurons in three clusters based on electrophysiological criteria. Cluster I neurons are identified by their irregular firing pattern and steeply rising, monophasic spike AHPs; cluster II cells are identified by their regularity in spontaneous firing and biphasic AHPs and cluster III cells by an unusually large rebound depolarization after a hyperpolarizing current pulse (Pennartz et al. 1998). Thus our second comparison was to determine which of these clusters comprise VP cells. Of 17 VP cells, 16 (94%) were classified as cluster I cells, and one cell possibly presented a case intermediate between cluster I and II. In line with this finding, none of the cluster II and III cells ($n = 5$) in slices stained for neurophysin turned out positive.

DISCUSSION

This study provides the first analysis of membrane properties and single-cell morphology of VP neurons in the SCN. Our main conclusion is that VP cells are quite representative for a majority of SCN neurons. They exhibit a pronounced time-dependent rectification but no large rebound depolarization. Provided that the SFR was < 5 Hz, their spontaneous firing behaviour was irregular almost without exception. Single spikes were followed by monophasic AHPs and trains of spikes evoked by positive current injection revealed a moderate frequency adaptation and were followed by an AHP up to a few seconds in duration. Membrane potential

records at hyperpolarized (-80 mV) levels were often studied with spontaneous PSPs. When comparing these properties to non-VP cells in the dorsomedial SCN, no conspicuous differences were found. This indicates that SCN cells belonging to a specific peptidergic phenotype may not have a unique electrophysiological signature by which these cells can be identified during the recording. However, it cannot be ruled out that a subtle difference exists with non-VP cells that was not uncovered by the currently used current-clamp protocols.

When placing the present findings in the context of the proposed partitioning in three SCN cell clusters, all VP cells except one could be clearly allocated to cluster I, which constitutes the majority of neurons (63%) in visual patch-clamp recordings throughout the SCN. This result agrees with our previous finding that cluster II and III cells are located outside the VP-rich dorsomedial region of the SCN (Pennartz et al. 1998). When the result is taken into account that non-VP cells in the dorsomedial region also belong to cluster I, the conclusion emerges that VP cells form a subset of cluster I cells. Thus there is a fair probability, but no certainty, that a dorsomedially located cluster I cell is VP positive. Cluster II and III cells belong to a different, unknown peptidergic phenotype. The lack of distinction between dorsomedial VP and non-VP cells stands in contrast to the difference between VP and oxytocin neurons in the supraoptic nucleus, which holds that oxytocin neurons display a time- and voltage-dependent outward rectification and associated rebound depolarization that are lacking in VP neurons (Stern and Armstrong 1995).

A point of careful consideration concerns the question whether the electrophysiological fragility of a number of VP neurons might be due to an unhealthy condition. We argue that this is most probably not the case because of three reasons. First, shortly after membrane rupture VP cells generated high, narrow spikes, whereas rundown was only seen to occur later, probably as a consequence of whole cell dialysis. Indeed, in voltage-clamp recordings we noted gradual rundown of a tetraethylammonium-sensitive outward rectifying K^+ current that may be important for spike repolarization and deinactivation of Na^+ channels (De Jeu et al., unpublished observations). Second, the preparation and maintenance conditions in this study were identical to those in a previous one (Pennartz et al. 1998), in which many neurons were recorded without obvious rundown. Third, preliminary perforated patch recordings in the dorsomedial area of SCN revealed a highly similar profile of membrane properties as described here albeit that the spike amplitude and spike trains did not exhibit rundown.

The electrophysiological profile as described above raises several suggestions as to how synaptic inputs to VP cells may be converted into spike output. Due to their high-input resistance, VP cells, like many other cell types in SCN, are very sensitive to excitatory and inhibitory synaptic inputs. As was described for SCN cells in general, many VP cells are almost continuously bombarded by spontaneous synaptic inputs, most of which are probably $GABA_A$ receptor mediated (Jiang et al. 1995; Kim and Dudek 1992; Thomson and West 1990). This combination of properties may result in a powerful, sometimes tonic, inhibition of spontaneous firing in VP cells by $GABAergic$ inputs (cf. De Jeu and Pennartz,

unpublished observations; Gribkoff et al. 1997). The sources of such inputs are unknown but may lie at least in part within the SCN (Buijs et al. 1994; Strecker et al. 1997; Van den Pol 1991). Despite the fact that many cells receive these inputs, they are able to maintain a considerable SFR. Spontaneous firing may be continuously promoted because the membrane potential of VP cells traverses a voltage domain in which a slow component of Na^+ current can be readily activated (Pennartz et al. 1997). This component underlies spontaneous depolarizing ramps (slow spike prepotentials) that were also widely observed in VP cells (Fig. 1). Thus the cells possess at least one intrinsic excitatory mechanism that opposes inhibitory synaptic inputs. In turn, the frequency of occurrence and rising speed of the depolarizing ramps depend on the tonic membrane potential. Although it is not yet clear which conductances maintain the membrane potential, these experiments suggest the presence of a Ba^{2+} -sensitive K^+ current in VP neurons. This current has been shown to make a tonic, hyperpolarizing contribution to the membrane potential (De Jeu et al., unpublished observations).

What type of electrophysiological behavior may give rise to VP release from axon terminals? Traditionally, peptide release is considered to occur under regimens of high-frequency stimulation (~ 5 – 30 Hz) (Jan and Jan 1982). In VP neurons of the SCN, we observed that the maximal firing rate achieved just after onset of a spike train evoked by intracellular current injection was 35 Hz on average. This suggests that endogenous VP release from the SCN should occur below this value. Furthermore, our findings show that VP cells do not spontaneously fire in high-frequency bursts and reach SFRs ranging from 0 to 13 Hz. This range agrees with the SFRs reported for SCN neurons in general (cf. Gillette 1991; Groos and Hendriks 1979; Shibata et al. 1984) and hence it can be speculated that endogenous VP would be released at firing rates at least in the upper portion of this range. The presence of a circadian rhythm in SFR in VP neurons was not addressed in the current paper because expression of rhythmicity seems to be compromised by intracellular dialysis associated with the whole cell procedure (Pennartz et al. unpublished observations). However, it is of note that loose-patch recordings from VP cells confirmed the presence of a strong rhythm in firing rate, suggesting an electrophysiological substrate for rhythmic patterns of VP release (M.L.H.J. Hermes, N.P.A. Bos, and R. M. Buijs, unpublished observations).

With respect to their morphology, VP cells have few dendrites, and these dendrites are sparsely branched, indicating that the membrane area available for synaptic contact is less extensive than in, for example, striatal or cortical neurons. They have intra- as well as extranuclear axon collaterals, and both types of collateral were occasionally found to originate from the same VP neuron. Thus single VP cells have the capacity to direct spike output to other SCN cells, possibly constituting elements of the biological clock (cf. Castel et al. 1990; Van den Pol and Gorcs 1986) and to neurons in target regions that translate circadian output into rhythms in physiological functions such as corticosterone release (Kalsbeek and Buijs 1996). By consequence, a spike-driven rhythm in VP release (Earnest et al. 1991) may not only reach these "effector" areas but may also modulate circadian rhythmicity within the SCN itself. Indeed, findings by

Ingram et al. (1996) suggest that VP may amplify the rhythm by an excitatory effect during the day phase.

Another widely occurring feature of VP cells is the presence of dendritic varicosities. Electron microscopic observations support the possibility of dendritic transmitter release from these sites (Castel et al. 1996; Güldner and Wolff 1974). It is tempting to speculate that VP cells may therefore release transmitter by two different modes; on the one hand they control more remote targets via axons and synaptic boutons, and on the other hand dendritic release may modulate excitability of cells in the immediate vicinity of the neuron, either by discrete contacts or in a diffuse manner.

In conclusion, VP cells form a subset of cluster I cells, which are widely found throughout the SCN and are characterized by irregular firing and monophasic spike AHPs. Further properties of VP cells include time-dependent inward rectification, moderate frequency adaptation, and a Ba^{2+} -sensitive K^+ current. Their morphology suggests a limited capacity for dendritic integration of synaptic inputs and supports the possibility of dendritic transmitter release. More distant targets of a VP neuron may be controlled by a relatively small network of intra- and extra-SCN axon collaterals. Important questions to be addressed in the future concern the electrophysiological mechanisms underlying the circadian rhythm in VP release and the sources of excitatory or inhibitory input to VP cells.

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