Pergamon

www.neuroscience-ibro.com

PII: S0306-4522(01)00346-3

Neuroscience Vol. 107, No. 1, pp. 99–108, 2001

0306-4522/01 $20.00+0.00

MELATONIN GENERATES AN OUTWARD POTASSIUM CURRENT IN RAT SUPRACHIASMATIC NUCLEUS NEURONES *IN VITRO* INDEPENDENT OF THEIR CIRCADIAN RHYTHM

M. VAN DEN TOP,[a,b]* R. M. BUIJS,[a] J. M. RUIJTER,[c] P. DELAGRANGE,[d] D. SPANSWICK[b] and M. L. H. J. HERMES[a]

[a]Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands

[b]Department of Biological Sciences, Neurobiology group, Gibbet Hill Campus, University of Warwick, Coventry CV4 7AL, UK

[c]Department of Anatomy and Embryology, Academic Medical Centre, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

[d]Institut de Recherches Internationales Servier, 92415 Courbevoie Cedex, France

Abstract—The present study investigated the membrane mechanisms underlying the inhibitory influence of melatonin on suprachiasmatic nucleus (SCN) neurones in a hypothalamic slice preparation. Perforated-patch recordings were performed to prevent the rapid rundown of spontaneous firing rate as observed during whole cell recordings and to preserve circadian rhythmicity in SCN neurones. In current-clamp mode melatonin (1 μM or 1 nM) application, in the presence of agents that block action potential generation and fast synaptic transmission, resulted in a membrane hyperpolarisation accompanied with a decrease in input resistance in the majority of SCN neurones (71–86%). The amplitude of the hyperpolarisation was not found to be significantly different between circadian time 5–12 and 14–21. In voltage-clamp mode melatonin (1 μM or 1 nM) induced an outward current accompanied with an increase in membrane conductance. The current was found to be mainly potassium driven with voltage kinetics resembling those of an open rectifying potassium conductance. Investigations into the signal transduction mechanism revealed melatonin-induced inhibition of SCN neurones to be sensitive to pertussis toxin but independent of intracellular cAMP levels and phospholipase C activity.

The present study shows that melatonin, at night-time physiological concentrations, reduces the neuronal excitability of the majority of SCN neurones independent of the time of application in the circadian cycle. Thus *in vivo* melatonin may be important for circadian time-keeping by amplifying the circadian rhythm in SCN neurones, by lowering their sensitivity to phase-shifting stimuli occurring at night.

Key words: hyperpolarisation, open rectifier, pertussis toxin-sensitive.

The suprachiasmatic nucleus (SCN) in the anterior hypothalamus contains the pacemaker that generates circadian rhythms (i.e. rhythms in cycles of about 24 h) in mammalian physiology and behaviour (Klein et al., 1991). The every-day adjustment of the phase of circadian rhythms to maintain a stable relationship to the 24-h environmental light/dark cycle is realised by a time-dependent phase-shifting influence of light on the pacemaker rhythm (Daan and Pittendrigh, 1976). Whereas light, acting through a retino-hypothalamic tract that directly innervates SCN, appears to play a major role in the so-called entrainment of circadian rhythms, other influences also contribute. One of these is melatonin (MLT), the major hormone of the pineal gland produced and secreted exclusively during the night (Vanecek, 1998). Timed injections of MLT entrain mammalian circadian rhythms in the absence of photic cues (Redman et al., 1983; Cassone, 1990). Since high-affinity binding sites for MLT are concentrated in SCN, it is believed that the pineal hormone has this effect through a direct influence on the circadian pacemaker (Vanecek et al., 1987; Weaver et al., 1989). This idea is supported by observations of an acute depression by MLT of the electrical activity of individual rat SCN neurones that are isolated in an *in vitro* hypothalamic slice preparation (Mason and Brooks, 1988; Shibata et al., 1989; Stehle et al., 1989). Furthermore, in similar slice preparations a shift in the phase of the circadian rhythm

*Correspondence to: M. van den Top, Department of Biological Sciences, Neurobiology group, Gibbet Hill Campus, University of Warwick, Coventry CV4 7AL, UK. Tel.: +44-2476-528368; fax: +44-2476-523701.

E-mail address: mvandentop@bio.warwick.ac.uk (M. van den Top).

Abbreviations: 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; ACSF, artificial cerebrospinal fluid; BMC, bicuculline methochloride; CT, circadian time; EGTA, ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid); MLT, melatonin; NBQX, 6-nitro-7-sulphamoylbenzo(*f*)-quinoxaline-2,3-dione; PLC, phospholipase C; PTX, pertussis toxin; RT-PCR, reverse transcription-polymerase chain reaction; SCN, suprachiasmatic nucleus; SFR, spontaneous firing rate; Sp-cAMPS, Sp-adenosine 3′5′-cyclic monophosphothioate triethylamine; TTX, tetrodotoxin; U-73122, 1-[6-9[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]aminohexyl]-1H-pyrrole-2,5-dione.

in electrical activity of rat SCN neurones is induced by timed MLT applications (McArthur et al., 1991; Starkey et al., 1995).

Until recently it was assumed that the inhibition of electrical activity was part of the cellular mechanism underlying the phase-shifting influence of MLT. However, in mice having a targeted deletion of the mt_1 receptor, a receptor responsible for all high-affinity binding of MLT in SCN, the inhibitory effect of MLT was abolished, yet the phase-shifting influence remained intact (Reppert et al., 1994; Liu et al., 1997). Although these results suggest that the cellular inhibition is not related to phase-shifting, it may still play an important role in circadian time (CT)-keeping by defining sensitivity of SCN neurones to phase-shifting stimuli occurring during darkness (Liu et al., 1997).

In the present study we examined the membrane mechanisms underlying MLT-induced inhibition of SCN neurones. We employed perforated-patch recording methods, to preserve spontaneous firing rate (SFR) and circadian rhythms in membrane properties. A preliminary account of these findings has been reported in abstract form (van den Top et al., 1998).

EXPERIMENTAL PROCEDURES

Slice preparation

30–60 day old male Wistar rats (obtained from Harlan, Horst, The Netherlands) were kept on a 12/12-h light/dark cycle (lights on at 07.00 am) for at least 3 weeks before use. Food and water were provided *ad libitum*. In accordance with national guidelines, rats were decapitated without anaesthesia between 09.00 and 11.00 am for recording at CT 5–12 (CT 0 is 07.00 am), and between 06.00 and 07.00 pm for recording at CT 14–21. Brains were rapidly removed from the cranial cavity and immediately placed in freshly prepared, oxygenated (95% O_2, 5% CO_2), ice-cold artificial cerebrospinal fluid (ACSF). Transverse slices (400–500 μm thick) were sectioned from hypothalamic blocks using a vibratome (Intracell Series 1000, Royston, UK). Slices were stored in a beaker containing oxygenated ACSF at 33 ± 1°C for at least 1 h before transfer to the recording chamber.

Solutions and drugs

The ionic composition of the ACSF was (in mM): 119.0 NaCl, 3.2 KCl, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 10.0 glucose, 1.3 $MgCl_2$, 2.4 $CaCl_2$. The ACSF had a pH of 7.3–7.4 and an osmolality of 295–300 mOsm/kg. ACSF containing 16 mM K^+ was prepared by substituting equimolar amounts of NaCl with KCl. The ionic composition of the recording solution was (in mM): 140.0 potassium gluconate, 10.0 HEPES, 10.0 KCl, 1.0 EGTA, and had a pH of 7.2–7.4 and an osmolality of 285–295 mOsm/kg. Both the polypeptide antibiotic gramicidin and the polyene antibiotic amphotericin-B were added to this solution as perforating substances, to final concentrations of 5 μg/ml and 250 μg/ml, respectively (Rae et al., 1991; Kyrozis and Reichling, 1995). Drugs used in the study included 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), MLT and Sp-adenosine 3′,5′-cyclic monophosphothioate triethylamine (Sp-cAMPS) from Research Biochemicals, Natick, MA, USA; 4-aminopyridine, amphotericin-B, barium chloride, cesium chloride, gramicidin, pertussis toxin (PTX), quinine, tolbutamide and U-73122 (1-[6-9[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]aminohexyl]-1-H-pyrrole-2,5-dione) from Sigma, Zwijndrecht, The Netherlands; bicuculline methochloride (BMC) and 6-nitro-7-sulphamoylbenzo(*f*)-quinoxaline-2,3-dione (NBQX) from Tocris Cookson, UK; and tetrodotoxin (TTX) from Alomone Lab., Israel. Drugs that were insoluble in water were dissolved in 100% dimethylsulphoxide (DMSO) and then further diluted in ACSF. The maximum concentration of DMSO applied to the brain slice was 0.05%, which did not influence SFR, resting membrane potential, or the holding current to clamp SCN neurones at −40 mV.

Drugs were bath-applied from reservoirs connected to the ACSF flow line by manually operable three-way valves. According to radio-immunoassay measurements, brief (1–2 min) applications of MLT at a concentration of 1 μM resulted in an approximate 30% concentration reduction before reaching the slice. Bath applications for prolonged periods of time (10–20 min) were assumed to result in effective concentrations in the recording chamber close to those present in the reservoirs.

Recording and data analysis

Recordings were obtained from submerged slices that were superfused with oxygenated ACSF at 33 ± 1°C, at a continuous flow rate of 5–8 ml/min using a gravitational perfusion system. The 'blind' patch-clamp recording method was applied (Blanton et al., 1989), using patch pipettes pulled from thin-walled borosilicate glass capillaries (GC150T-10, Clark Electromedical Instruments, UK) with a resistance of 4–7 MΩ when filled with recording solution. All recordings were made using an Axopatch-1D amplifier (Axon Instruments, USA). The series resistance of the perforated-patch recordings stabilised at 30–40 MΩ 15–20 min following seal formation and showed little variation over 1–2 h of recording. Recordings were terminated when series resistance increased by more than 15%. Current and voltage data were displayed on-line on a digital oscilloscope (Tektronix, USA) and stored on videotape with the use of an A–D converter (Neurodata, Pasadena, CA, USA). The pClamp 6.02 suite of programs and Axotape 2.0 (Axon Instruments) were used for data analysis off-line.

The effect of MLT on the SFR of SCN neurones was assessed in slices superfused with normal ACSF. The membrane mechanisms underlying this MLT influence were investigated in current-clamp and voltage-clamp mode in ACSF containing TTX (0.5 μM) to block action potentials, and BMC (10 μM) and NBQX (2 μM) to block $GABA_A$ and non-*N*-methyl-D-aspartate receptor-mediated fast synaptic potentials, respectively. In current-clamp mode resting membrane potentials were calculated from representative 1-min periods (sampled at 200 Hz). The recorded membrane potentials were not corrected for the possible occurrence of a Donnan potential as a result of the slight Cl^- permeability of the pores formed by amphotericin-B (Kyrozis and Reichling, 1995). Input resistances were measured from the instantaneous voltage deflections induced by negative current injections (amplitude 50 pA, duration 2 s). Input resistances in the presence of MLT were measured at control levels of resting membrane potential following manual clamping of SCN neurones. In voltage-clamp mode, neurones were clamped at a holding potential of −40 mV and the series resistance compensated by 60–80%; current signals were filtered at 1 kHz using a four-pole low-pass Bessel filter. Holding currents were calculated from representative 1-min periods (sampled at 200 Hz). The membrane conductance was calculated from the steady-state holding current values at −40 mV to −55 mV obtained from voltage–current relationships.

Theoretical voltage–current relationships of currents were determined with the Goldman, Hodgkin and Katz (G–H–K) equation (Goldman, 1943; Hodgkin and Katz, 1949).

G–H–K equation:

$$I_s = P_s Z_s^2 \left(\frac{VF^2}{RT}\right)\left(\frac{[S]_i - [S]_o \exp(-z_s VF/RT)}{1-\exp(-z_s VF/RT)}\right)$$

P_s = membrane permeability for K^+; $[S]_i$ and $[S]_o$ = internal and external potassium concentration, respectively; z = ionic charge; F = Faraday's constant; R = gas constant; T = absolute temperature; V = holding potential.

Neurones were recorded from all anatomical subdivisions (i.e. ventrolateral and dorsomedial aspects) of SCN. Cluster analysis

Table 1. Summary of circadian rhythm in membrane properties of rat SCN neurones recorded using perforated-patch-clamp methods

Parameter	CT 5–12	CT 14–21	Significance
SFR (Hz)	6.4 ± 0.4 (45)	0.1 ± 0.1 (10)	$P < 0.01$
Resting potential (mV)	−39.2 ± 1.4 (18)	−49.5 ± 2.3 (9)	$P < 0.01$
Holding current (pA)	−8.6 ± 2.9 (22)	20.8 ± 4.8 (11)	$P < 0.01$
Membrane conductance (nS)	1.05 ± 0.10 (14)	2.09 ± 0.18 (11)	$P < 0.01$

SFRs were recorded from cells in standard ACSF while resting membrane potential, holding current (at −40 mV) and membrane conductance were obtained in current- or voltage-clamp mode in ACSF containing TTX/BMC/NBQX. Values are expressed as mean ± S.E.M. with the number of cells in parentheses. Significant differences between CT 5–12 and CT 14–21 were observed in all parameters using independent two-tailed Student's *t*-tests.

of a population of cells randomly selected from our data ($n = 9$) revealed that all of the previously described clusters (1–3) of SCN neurones were included in the study (Pennartz et al., 1998).

Statistical analysis

The two-tailed Student's *t*-test for dependent samples was used to determine statistically significant ($P < 0.05$) differences between two related populations. For non-related experimental groups the two-tailed Student's *t*-test for independent samples was applied. All statistics were performed on a personal computer running Statistica (Statsoft).

RESULTS

Perforated-patch recording preserves the circadian rhythm in membrane properties of SCN neurones

SCN neurones recorded at CT 5–12 or CT 14–21 with the perforated-patch method maintained firing rates not significantly different from the frequency measured in cell-attached mode for periods exceeding 1 h (Fig. 1A). Mean SFRs of SCN neurones in both time periods (measured 20 min after seal formation) were comparable to those obtained with extracellular recording techniques and revealed a clear circadian rhythm in SFR (Fig. 1, Table 1) (Shibata et al., 1989; Starkey et al., 1995). Moreover, perforated-patch recordings from cells recorded in the presence of TTX, BMC and NBQX revealed circadian differences in several other membrane properties including resting membrane potential (in current-clamp mode), holding current to clamp cells at a membrane potential of −40 mV, and (in voltage-clamp mode) membrane conductance (Table 1). These results suggest that circadian rhythmicity in SCN neurones is preserved during perforated-patch recordings, although we acknowledge that given our small *n* values we may have sampled from different populations of neurones

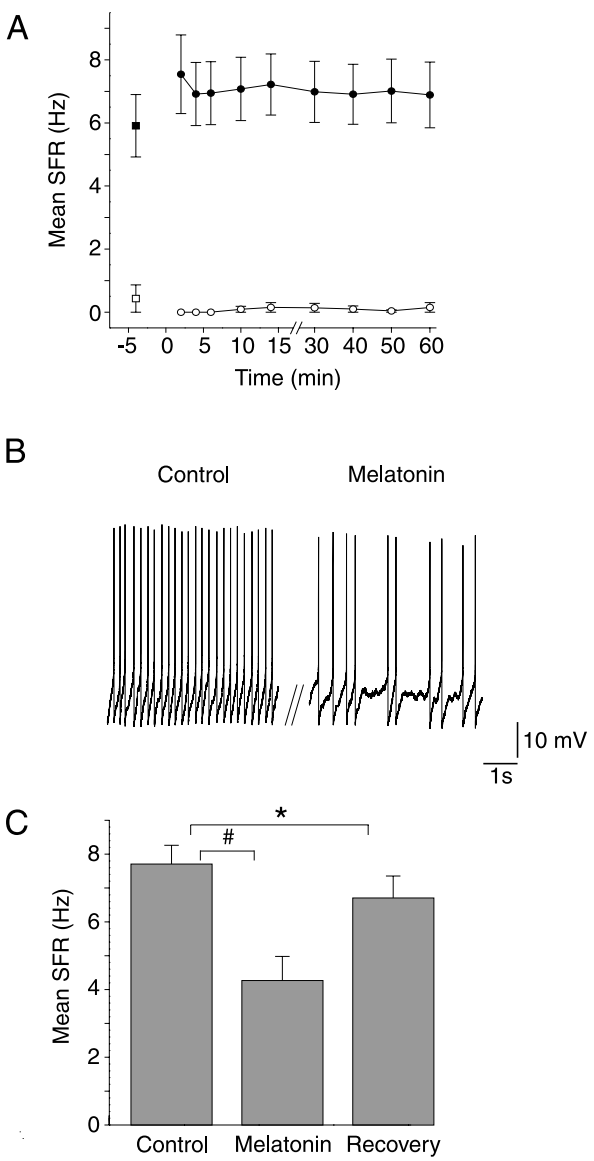

Fig. 1. Perforated-patch recording preserves spontaneous firing behaviour and MLT-induced inhibition of rat SCN neurones. (A) Mean (± S.E.M.) SFR of SCN neurones at CT 5–12 (●, $n = 8$) and CT 14–21 (○, $n = 7$) recorded over a 1-h period at 33°C using the perforated-patch recording technique. The cell-attached recordings (denoted with ■ and □) were obtained from an independent set of experiments performed under similar conditions but using pipette solutions lacking gramicidin and amphotericin-B. At any time during the 1 h of recording the mean firing rate (sampled over 1-min periods) was not significantly different ($P \geq 0.32$; independent two-tailed Student's *t*-tests) from the mean values obtained in a cell-attached configuration (B) Typical example of the response of a MLT-sensitive neurone to a brief 1-min bath application of MLT (1 μM) at CT 5–12. Traces were recorded, using a sampling rate of 5 kHz, 1 min prior to and 10 min following MLT application (// 10-min break). (C) Bar graph showing the mean (± S.E.M.) MLT-induced decrease in firing rate as observed in the 15 responsive SCN neurones ($^{\#}P < 0.01$). Total recovery to control values was not obtained following prolonged periods (> 30 min) of wash-out ($^{*}P < 0.05$).

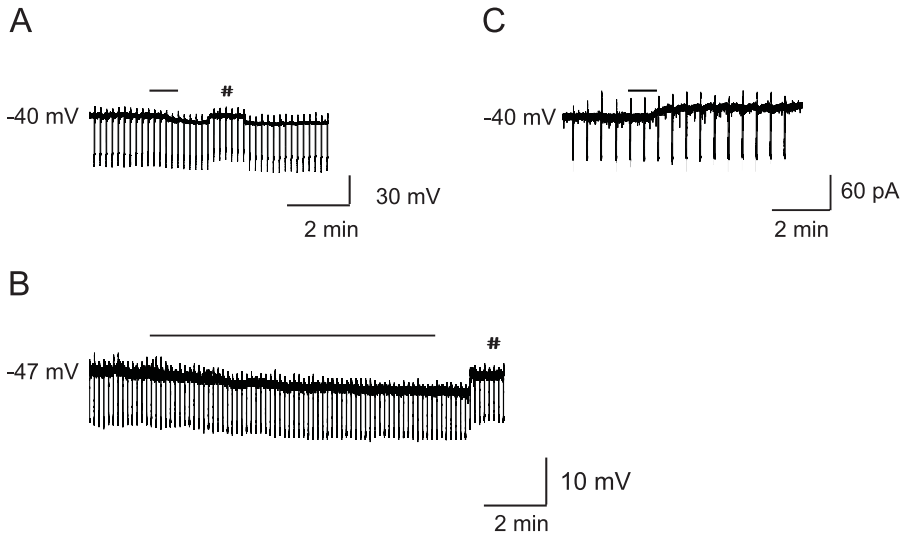

Fig. 2. MLT-induced hyperpolarisation and outward current in rat SCN neurones as obtained in current- and voltage-clamp, respectively. (A) Current-clamp recording illustrating the membrane hyperpolarisation and decrease in input resistance induced by a brief 1-min application of MLT (1 μM) (indicated by the line above the trace) to a SCN neurone at resting membrane potential. At the point indicated (#) the membrane potential was manually clamped back to control values, by injection of constant current, to illustrate the decrease in input resistance (apparent from the reduced membrane potential deflections in response to hyperpolarising current pulse injections of 50 pA, into the cell). (B) Current-clamp recording (CT 14–21) illustrating a membrane hyperpolarisation induced by MLT 1 nM administration for 10 min. Note the difference in the kinetics of the response in comparison to MLT applied at a 1 μM concentration (see A). # Indicates the part of the trace where constant current was injected to clamp the membrane potential back to pre-response values to show the decrease in input resistance associated with the MLT-induced response (current step −20 pA). The line above the traces illustrates the timing and duration of MLT administration. (C) Under similar conditions application of MLT (1 μM) to a SCN neurone clamped at a holding potential of −40 mV (in voltage-clamp mode) induced an outward current with a concurrent increase in membrane conductance (as indicated by the increased current amplitudes in response to constant voltage steps to −80 mV). The line above the traces illustrates the timing and duration of the MLT application.

across CT 5–12 and CT 14–21. Our findings are in agreement with an earlier report (De Jeu et al., 1998). In contrast, a previous study reported a rundown of SCN neurones during whole cell recordings resulting in a loss of SFR and circadian rhythmicity (Schaap et al., 1999). For this reason the present study was performed using the perforated-patch-clamp recording technique.

MLT decreases day-time SFR of SCN neurones

Brief 1- or 2-min bath applications of MLT (1 μM) at CT 5–12 resulted in a decrease in SFR in 15/32 (46.9%) SCN neurones (Fig. 1B). The SFR was significantly reduced from 7.7 ± 0.6 Hz in control conditions to 4.3 ± 0.7 Hz following MLT administration ($n = 15$, $P < 0.01$; Fig. 1C). The effect was fully expressed within 3 min of the start of application, and only partially recovered following prolonged (> 30 min) wash-out periods. 2/32 SCN neurones (6.3%), having a mean SFR of 4.7 ± 0.7 Hz in control conditions, responded to MLT application with an increase in SFR to 6.4 ± 0.3 Hz.

Table 2. Summary of data comparing the effects of different concentrations of MLT on SCN neurones at different CTs

	CT 5–12	CT 14–21
Control (mV)	−42.1 ± 2.4 (12)	−47.2 ± 2.2 (5)
MLT (1 μM) (mV)	−48.7 ± 2.5 (12)	−52.6 ± 1.6 (5)
Δ	−6.7 ± 0.7 (12)	−5.4 ± 1.1 (5)
Control (mV)	−39.3 ± 5.8 (5)	−48.4 ± 1.5 (3)
MLT (1 nM) (mV)	−44.0 ± 6.4 (5)	−50.7 ± 1.5 (3)
Δ	−4.8 ± 1.3 (5)	−2.4 ± 1.4 (3)

Resting membrane potentials are means ± S.E.M. and the number of cells is indicated in parentheses. Δ denotes the difference in membrane potential as observed between recordings obtained in the absence or presence of MLT.

MLT hyperpolarises the majority of SCN neurones at CT 5–12 and 14–21

In ACSF containing TTX, BMC and NBQX, brief applications of MLT (1 μM) at CT 5–12 induced a hyperpolarisation of the membrane potential by 6.7 ± 0.7 mV in 12/14 (85.7%) SCN neurones reaching a new equilibrium within 3 min of the onset of application (Fig. 2A, Table 2). Prolonged 10–20-min applications of MLT, at a concentration of 1 nM, were effective in 5/7 (71.4%) neurones, inducing a mean membrane hyperpolarisation of 4.8 ± 1.3 mV that peaked within 15 min of the onset of application (Fig. 2B, Table 2). Injection of hyperpolarising rectangular-wave current pulses indicated that the membrane hyperpolarisation induced by 1 μM MLT was accompanied with a decrease in input resistance (Fig. 2A) from 942 ± 79 MΩ to 840 ± 70 MΩ ($n = 8$).

MLT (1 μM) applied at CT 14–21 induced a mean membrane potential hyperpolarisation of 5.4 ± 1.1 mV in 5/7 (71.4%) neurones, an amplitude not significantly different from the mean hyperpolarisation induced by MLT (1 μM) administration at CT 5–12 ($P = 0.33$)

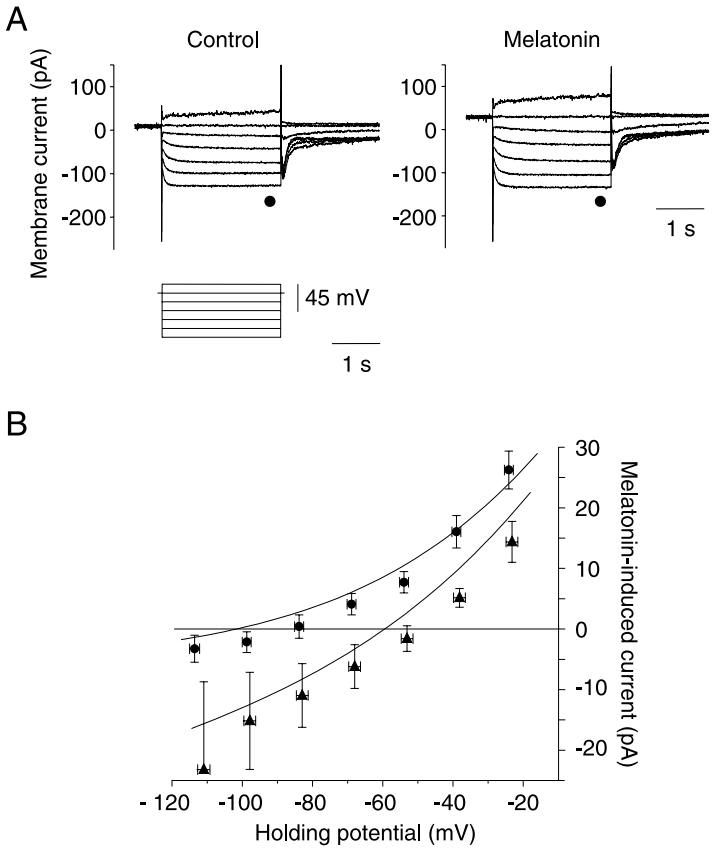

Fig. 3. The MLT-induced outward current is mediated by an outward conductance that resembles an open rectifying potassium conductance. (A) Superimposed plots of the membrane currents in SCN neurones induced by injecting a range of hyperpolarising and depolarising voltage steps from a holding potential of −40 mV (−25 to −115 mV: 15 mV increment). The protocol is indicated below. Subtraction of the steady-state membrane currents (measured at ●) in control conditions from those following brief 1-min applications of MLT (1 μM) gives the MLT-induced current at different membrane potentials (as plotted in B). (B) The mean (±S.E.M.) MLT-induced current in ACSF containing 3.2 mM (●) and 16 mM (▲) potassium. The horizontal error bars are the S.E.M. from the mean holding potential, calculated after correction for bath offset potentials. In 3.2 mM potassium, the MLT-induced current reversed at −87.1 mV and demonstrated outwardly rectifying properties. In 16.0 mM potassium, the reversal potential was shifted positive by 36 mV to a value of −51.1 mV, near the calculated shift of 42.4 mV predicted for a current selectively carrying potassium. The continuous lines display the theoretical voltage–current relationships of open rectifying potassium currents with these extracellular potassium concentrations, as determined by the G–H–K equation (see Experimental procedures). The recorded outward currents were well fitted by the G–H–K equation ($R^2 = 0.69$ and 0.40 for 3.2 and 16.0 mM external potassium, respectively).

(Table 2). MLT (1 nM) application at CT 14–21 hyperpolarised 3/4 (75.0%) neurones with a mean value of 2.4 ± 1.4 mV, an amplitude not significantly different ($P = 0.27$) from the mean hyperpolarisation induced by MLT (1 nM) administration at CT 5–12 (Fig. 2B, Table 2). Subsequent application of a higher concentration of MLT (1 μM) to these cells was used to verify sensitivity to MLT which induced a 4.6 ± 0.7 mV ($n = 3$) membrane hyperpolarisation.

MLT generates an outward current in the majority of SCN neurones irrespective of the CT of application

In voltage-clamp mode, brief applications of MLT (1 μM) at CT 5–12 generated an outward current in 13/15 (86.7%) neurones held clamped at a holding potential of −40 mV (Fig. 2C). The mean peak amplitude of the outward current was 15.5 ± 2.1 pA and was accompanied by a 52.3 ± 8.6% increase in the steady-state membrane conductance. MLT at a lower concentration of

Table 3. The magnitude of the outward current and change in membrane conductance induced by MLT in rat SCN neurones is not dependent on the CT of application

	CT 5–12	CT 14–21
MLT 1 μM		
ΔI_{hold} (pA)	15.5 ± 2.1 (13)	11.3 ± 2.4 (7)
ΔG_{cell} (nS)	0.47 ± 0.07 (12)	0.53 ± 0.09 (7)
MLT 1 nM		
ΔI_{hold} (pA)	10.8 ± 1.2 (7)	7.7 ± 1.8 (6)
ΔG_{cell} (nS)	0.36 ± 0.07 (5)	0.30 ± 0.06 (6)

The MLT-induced current (ΔI_{hold}) was calculated by subtracting the holding current at −40 mV, prior to and following MLT application. Membrane conductance was calculated using a −15 mV step, from −40 mV to −55 mV in voltage–current relationships, and subtracting the steady-state holding current values. The MLT-induced increase in membrane conductance is expressed as ΔG_{cell}. Values are expressed as mean ± S.E.M. with the number of cells in parentheses.

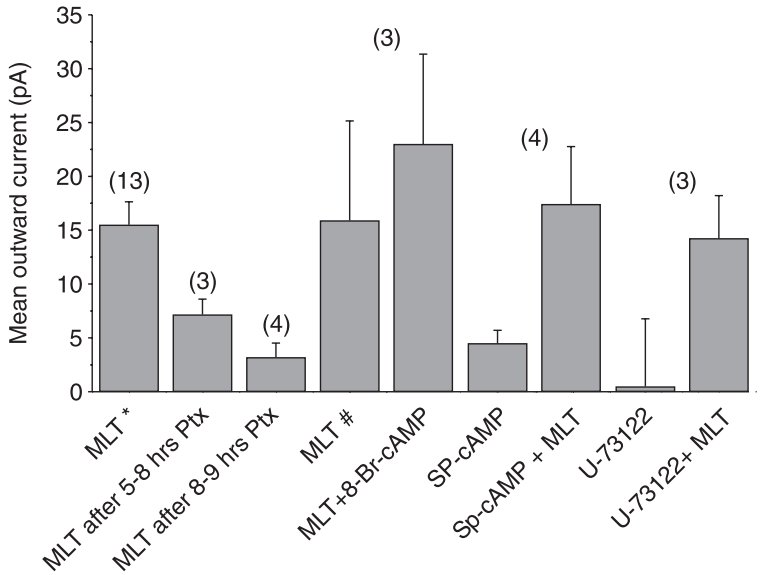

Fig. 4. The MLT-induced outward current is PTX-sensitive but independent of cAMP levels and PLC activity. Mean MLT-induced outward current (±S.E.M.) in the absence or presence of drugs that modulate second messenger systems related to the activation of PTX-sensitive G-protein-coupled receptors. MLT* = control values from a different set of experiments; MLT# = outward current obtained from internal control. Bars are in order of application for control and MLT in the drug-manipulated MLT response. All groups were obtained from different sets of recordings. The numbers of experiments performed per group are denoted in parentheses above the individual bars.

1 nM induced an outward current of peak amplitude 10.8 ± 1.2 pA and a concomitant 25.2 ± 5.7% increase in membrane conductance in 7/8 (87.5%) SCN neurones.

At CT 14–21, MLT (1 μM) induced an outward current in 7/9 (77.8%) cells. The response and absolute increase in steady-state membrane conductance were not significantly different from those generated by MLT (1 μM) at CT 5–12 ($P = 0.23$ and 0.63, respectively). Similarly, prolonged 1 nM MLT applications at CT 14–21 induced responses in 6/8 (75.0%) neurones that were not significantly different from those following 1 nM applications at CT 5–12 ($P = 0.16$ and 0.52, respectively). A summary of these results is given in Table 3.

MLT activates an open rectifying potassium current

The voltage dependence of the MLT current was determined by calculating the difference in steady-state membrane current at different membrane potentials prior to and following 1 μM MLT applications to induce maximal responses (Fig. 3A). The reversal potential of the MLT-induced current, obtained by fitting the linear part of the curve between −40 and −100 mV ($R^2 = 0.94$), was −87.1 mV, a value 15 mV more positive than the predicted reversal potential for potassium of −102.0 mV under our recording conditions (Fig. 3B). The current displayed outwardly rectifying properties: at holding potentials depolarised to the reversal potential it appeared disproportionally enhanced, whereas at holding potentials negative to the reversal potential it was attenuated (Fig. 3B). A five-fold increase in the ACSF potassium concentration, to 16.0 mM, induced a 36.0 mV shift in the reversal potential to −51.1 mV, calculated by fitting the linear part of the curve between −40 and −100 mV ($R^2 = 0.99$) (Fig. 3B). As with the control reversal, the reversal in 16 mM extracellular potassium was more positive (around 8 mV) than the predicted reversal potential of −59.6 mV for a potassium-selective current. The increase in extracellular potassium concentrations visibly diminished the outwardly rectifying properties of the MLT current, suggesting that the rectifying properties of the MLT-induced current are due to unequal permeant ion concentrations across the cellular membrane (Fig. 3B). In agreement with this, the G–H–K current relation (Goldman, 1943; Hodgkin and Katz, 1949) fitted the MLT-induced outward current in 3.2 and 16.0 mM external potassium well with R^2 values of 0.69 and 0.40, respectively. However, the theoretical curves were positioned at slightly more negative membrane potentials than the MLT-induced current curves (Fig. 3B).

The MLT-induced outward current is barium-sensitive

To further determine the characteristics of the MLT-induced current we assessed the influence of several potassium channel blockers on the amplitude of the MLT-induced current in the presence of TTX/BMC/NBQX. To avoid ambiguity regarding the effectiveness of agents to block the MLT-induced current we only used cells showing a control response to MLT (1 μM) larger than 10 pA. We judged this not to bias our results as the current voltage characteristics of the MLT-induced outward current were independent of the amplitude of the response (data not shown). Tolbutamide (50 μM), which blocks ATP-sensitive potassium channels, reported to be present in SCN (Hall et al., 1997), was ineffective ($n = 4$, $P = 0.33$). Also quinine (100 μM), known to inhibit various types of potassium channels, including non- or weakly rectifying tandem pore domain potassium channels (Lesage et al., 1996; Leonoudakis et

al., 1998), did not block the MLT-induced current ($n = 4$, $P = 0.22$). 4-Aminopyridine (1 mM), a blocker of the transient outward potassium current (or A-current) present in all SCN neurones (Rudy, 1988; Bouskila and Dudek, 1995), did not block the MLT-induced current ($n = 4$, $P = 0.21$). Cesium (1 mM), a non-selective blocker of the mixed cationic inward rectifier (I_h) observed in the majority of SCN neurones (Akasu et al., 1993; Pape, 1996), did not affect the MLT-induced current ($n = 5$, $P = 0.90$), whilst it clearly blocked I_h (data not shown). Only barium (500 μM), a blocker of the potassium-selective inward rectifier and some tandem pore domain potassium channels (Rudy, 1988; Akasu et al., 1993; Goldstein et al., 1996; Lesage et al., 1996), resulted in a significant 55% reduction in the MLT-induced current ($n = 5$, $P < 0.05$).

The MLT-induced current is PTX-sensitive and cAMP- and phospholipase C (PLC)-independent

To identify the MLT activated intracellular signal transduction mechanism we performed experiments in which slices were preincubated in PTX, a blocker of inhibitory (Gi) and other (Go) G-proteins. Voltage-clamp experiments in slices pretreated with PTX, 2 μg/ml for 3–5 and 8–9 h, induced an inhibition of the MLT-induced current. The MLT-induced current observed under control conditions was 15.5 ± 2.1 pA ($n = 13$), and was reduced depending on the duration of preincubation in PTX. MLT-induced currents were reduced relative to control by 54% ($n = 3$) and 79% ($n = 4$) following preincubation in PTX for 3–5 and 8–9 h, respectively (Fig. 4). In current-clamp mode PTX preincubation (5–8 h) resulted in a 70% inhibition of the MLT-induced hyperpolarisation, from 6.7 ± 0.7 mV ($n = 12$) in control to 2.0 ± 0.5 mV in PTX ($n = 7$). The decrease in MLT response observed is not due to a reduced viability of the slices as a result of PTX pretreatment as (i) experiments with slices preincubated for 5–8 h in the absence of PTX expressed normal sensitivity to MLT application in both current- and voltage-clamp under our recording conditions (data not shown) and (ii) membrane properties of the SCN neurones (SFR, membrane potential and input resistance) following PTX pretreatment were similar to control experiments.

The membrane-permeable non-hydrolysable cAMP analogue 8-Br-cAMP was used to investigate the dependence of the MLT-induced current on the inhibition of cAMP levels, as high-affinity MLT receptors are found to inhibit forskolin-stimulated cAMP levels (Reppert et al., 1994). Application of 500 μM 8-Br-cAMP ($n = 3$) following MLT (1 μM) induced a small additional outward current but was without effect on the MLT-induced current (Fig. 4). Moreover, the membrane-permeable non-hydrolysable protein kinase A (PKA) activator Sp-cAMPS (10 μM) applied for 20–30 min prior to MLT (1 μM) induced a small outward current but again was without effect on the MLT-induced current (Fig. 4). Application of the PLC inhibitor U-73122 (5 μM) for 20 min prior to MLT (1 μM) administration did not block the MLT-induced outward current ($n = 3$).

DISCUSSION

The present study characterised the membrane mechanisms underlying the previously reported depressing influence of MLT on the firing frequency of SCN neurones (Mason and Brooks, 1988; Shibata et al., 1989; Stehle et al., 1989). Perforated-patch recordings were employed to prevent the rapid and pronounced deterioration of the SFR of SCN neurones as reported during whole cell recordings (Schaap et al., 1999). Moreover, as perforated-patch recordings preserved circadian rhythmicity in SCN neurones (de Jeu et al., 1998), we judged this method the most suitable and appropriate to investigate a possible circadian modulation of the MLT-induced inhibition.

Using this technique we demonstrate that the majority of SCN neurones are sensitive to MLT, a feature that is independent of the CT of administration, and also detectable with low, physiological, concentrations of the hormone. In the present study MLT 1 nM application was deemed to represent a physiological concentration. Within the literature plasma and cerebrospinal concentrations of MLT at night have been reported to range between 0.1 and 0.6 nM in a range of species including primates (see Vanecek, 1998). In sheep MLT levels adjacent to the SCN in the third ventricle have been reported as high as 9 nM, 20-fold higher than observed in other parts of the ventricular system (Skinner and Malpaux, 1999). It is impossible to predict the bioavailability of MLT to SCN neurones based on these studies but the concentration is likely to be close to the 1 nM concentration used in the present study. Furthermore, it should be noted that in a previous study using hypothalamic slice preparations similar to ours, a phase shift in the electrical activity of SCN neurones could be induced by applying MLT over a period of 1 h at concentrations as low as 0.01 nM (McArthur et al., 1997). The present study did not perform experiments using concentrations of MLT as low as this as we were unable to perform and consistently sustain recordings that allow such long-term applications. Furthermore, precisely how well MLT penetrates tissues in slice preparations is impossible to know. Consequently, the concentration of MLT at the sites of receptor localisation in recorded cells in slice preparations is unknown. However, based upon this information, we feel it reasonable to suggest that the concentrations of MLT used in the present study are likely within the physiological range.

MLT application resulted in a membrane hyperpolarisation accompanied by a decrease in input resistance. Voltage-clamp experiments revealed MLT induced an outward current accompanied by a large increase in slope conductance. The voltage characteristics of the current appeared to be a reflection of the transmembrane concentration gradient for potassium as described by the G–H–K equation (Goldman, 1943; Hodgkin and Katz, 1949), hence mimicking the current generated by two-pore domain potassium channels, initially characterised in *Drosophila melanogaster* (Goldstein et al., 1996). The position at more negative membrane potentials of the theoretical voltage–current relationship of a potassium-

selective current suggests that a yet unidentified mechanism involving ions other than potassium may have made a minor contribution to the MLT-induced current. However, MLT had no effect on the hyperpolarisation-activated mixed cationic current I_h, in agreement with a lack of effect of the I_h blocker cesium on the MLT-induced current. Moreover, the observed shift cannot be attributed to an artefact related to space-clamp problems as this would result in a negative shift of the voltage–current relationship in contrast to the positive shift observed. In the present study membrane potentials were not compensated for possible Donnan potentials. However, as only partial wash-out of Cl^- occurs during perforated-patch recording performed using amphotericin-B (Kyrozis and Reichling, 1995), a Donnan related offset in the membrane potentials in the present study is expected to be minimal. Indeed, a previous study in SCN neurones did not observe a difference in membrane potential between recordings performed as described in the present study and recordings using only gramicidin which prevents Donnan potentials (Kyrozis and Reichling, 1995; de Jeu et al., 1998).

Two high-affinity MLT receptors have been identified in the SCN: (i) mt_1 receptors responsible for all high-affinity MLT binding in the SCN and suggested to mediate the MLT-induced decrease in the electrical activity of SCN neurones (Reppert et al., 1994; Liu et al., 1997); (ii) MT-2 receptors of which only the mRNA can be detected using reverse transcription-polymerase chain reaction (RT-PCR) (however see Wan et al., 1999). Both MLT receptors are coupled to an inhibitory G-protein (Gi) and activation results in an inhibition of forskolin-stimulated intracellular cAMP levels but not of basal cAMP levels (Reppert et al., 1994). We found the inhibitory influence of MLT on SCN neurones to be sensitive to PTX, in agreement with previous studies (Starkey et al., 1995; Liu et al., 1997), and consistent with activation of Gi. However, a lack of sensitivity of the MLT-induced current to intracellular cAMP levels and PKA activity observed in the present study suggests that MLT-induced inhibition of SCN neurones is mediated through a mechanism other than negative coupling to adenylate cyclase and inhibition of cAMP levels. In this instance, the signal transduction mechanism involved in the depressing influence of MLT on the electrical activity of SCN neurones may be mediated through a direct interaction between activated $G\alpha$ and/or $\beta\gamma$ subunits and the target ion channel. Such a signal transduction mechanism has been described in other systems (for review see Wickman and Clapham, 1995).

Comparison with previous results on the mechanism of action of MLT on SCN neurones shows two major differences. First, an earlier study reported a MLT-induced outward current in only a subpopulation (35%) of SCN neurones, and an inhibition of I_h contributing to the total outward current generated by MLT (Jiang et al., 1995). The discrepancy with the present results may, at least partially, be explained by cytoplasmic dialysis occurring with whole cell recording (Pusch and Neher, 1988) The present study reveals that perforated-patch recordings in contrast to whole cell recordings preserve spontaneous activity of SCN neurones. It is conceivable that mechanisms involved in the modulation of this spontaneous activity, such as MLT receptor-dependent signalling, are similarly affected. Moreover, the amplitude/kinetics of I_h in SCN neurones are dependent on intracellular cAMP levels (Akasu and Shoji, 1994). Hence wash-out of cAMP during whole cell recording might explain the small percentage of SCN neurones found to express I_h in the study by Jiang et al. (1995) (39%) in contrast to the present study (100%). However, wash-out of cAMP cannot explain the difference in cesium sensitivity of the MLT current nor the difference in the percentage of SCN neurones found sensitive to MLT in the two studies. Previous studies have also proposed that MLT receptors in CNS may be coupled to the potassium inwardly rectifying channels of the GIRK family (Nelson et al., 1996). Data presented in the present study, however, do not support this either. However, that MLT receptors may couple to the GIRK family of inwardly rectifying potassium channels was derived from experiments expressing these ion channels together with MLT receptors in *Xenopus* oocytes. It is therefore likely that the presence of different target ion channels in 'wild-type' SCN neurones will result in a different scenario.

The present study might have underestimated the inhibitory influence of MLT on SCN neurones as a recent study has shown that MLT increases the amplitude of $GABA_A$-mediated synaptic transmission in SCN (Wan et al., 1999). However, the frequency of GABAergic synaptics might decline as part of the GABAergic input originates from within the SCN and thus these neurones could be potentially inhibited by MLT (Strecker et al., 1997). It should also be noted that Jiang et al. (1995) did not observe an effect of MLT on GABAergic synaptics.

The present results, in contrast to previous studies using extracellular recording methods (Shibata et al., 1989; Stehle et al., 1989; but see Liu et al., 1997), demonstrate the lack of a particular 'time-window' of sensitivity for MLT to inhibit SCN neuronal electrical activity. It should be noted though that the different electrophysiological recording techniques used might result in a recording being made from different subtypes of SCN neurones and that the sample size used in studies using extracellular recording techniques is larger than the sample size in the present study. Furthermore, a recent study has shown that the mRNA of the mt_1 receptor does not show a circadian expression pattern as determined using RT-PCR (Sugden et al., 1999). Moreover, the lack of such a 'time-window' is in contrast to MLT's phase-shifting influence on SCN neurones (Cassone, 1990; McArthur et al., 1991). Thus the present results support the idea that the inhibition of SCN neurones is not part of the cellular mechanism underlying the phase-shifting influence of MLT (Liu et al., 1997). Data described in the present study also reveal a large increase in membrane conductance following MLT application at night using a physiological concentration of MLT. The circadian decrease in firing frequency during subjective night appears to be the result of a membrane mechanism

that resembles that induced by MLT. This is suggested by the fact that SCN neurones during subjective night have a hyperpolarised membrane potential and, when analysed in voltage-clamp mode, show an outward shift of the holding current with a concomitant increase in membrane conductance. The exact membrane mechanism underlying this circadian variation remains to be determined. The increased conductance induced by MLT at night could decrease the efficacy of excitatory synaptic input to change neuronal activity in SCN neurones hence preserving SCN night-time output. In this way MLT may help to maintain night-time homeostasis, as SCN output controls, for instance, the secretion of hormones like MLT and corticosterone through polysynaptic pathways (Larsen et al., 1998; Buijs et al., 1999). Furthermore, MLT may act as a gainsetter for excitatory synaptic input to shift the phase of the biological clock (see Liu et al., 1997). However, it was proposed that light exposure at night may not be subject to this gainsetting MLT modulation, since it results in an immediate decrease in plasma MLT levels (Liu et al., 1997). Still, the lack of a fast recovery of the MLT-induced effect observed in the present study suggests that an acute reduction of plasma MLT levels does not immediately result in a change of SCN membrane properties. This hypothesis is supported by the observation that mice incapable of MLT synthesis are more sensitive to the phase-shifting by night-time light exposure than mice that do have a normal pattern of MLT secretion (Von Gall et al., 1998).

CONCLUSION

We have observed that physiological concentrations of MLT decrease the excitability of SCN neurones independent of the timing of application in their circadian cycle. Moreover, the results obtained using a night-time physiological concentration of MLT (1 nM) at CT 14–21 are likely to mimic the responses observed *in vivo* at night. MLT activates a potassium current with outwardly rectifying properties at physiological internal and external potassium concentrations. The effect is prolonged and, although not involved in phase-shifting, likely to be significant for CT-keeping.

Acknowledgements—The authors wish to thank Dr M.F. Nolan for useful comments on the manuscript. This work was supported by a Grant from Institut de Recherches Internationales Servier (no. PHA SCREEN-907-NLD).

REFERENCES

Akasu, T., Shoji, S., 1994. cAMP-dependent inward rectifier currents in neurons of the rat suprachiasmatic nucleus. Pflüg. Arch. 429, 117–125.
Akasu, T., Shoji, S., Hasuo, H., 1993. Inward rectifier and low-threshold calcium currents contribute to the spontaneous firing mechanism in neurons of the rat suprachiasmatic nucleus. Pflüg. Arch. 425, 109–116.
Blanton, M.G., LoTurco, J.J., Kriegstein, A.R., 1989. Whole cell recording from neurons in slices of reptilian and mammalian cerebral cortex. J. Neurosci. Methods 30, 203–210.
Bouskila, Y., Dudek, F.E., 1995. A rapidly activating type of outward rectifier K^+ current and A-current in rat suprachiasmatic nucleus neurons. J. Physiol. 488, 339–350.
Buijs, R.M., Wortel, J., Heerikhuize, J.J., Feenstra, M.G.P., Ter Horst, G.J., Romijn, H.J., Kalsbeek, A., 1999. Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur. J. Neurosci. 11, 1535–1544.
Cassone, V.M., 1990. Effects of melatonin on circadian systems. Trends Neurosci. 13, 457–464.
Daan, S., Pittendrigh, C.S., 1976. A functional analysis of circadian pacemakers in nocturnal rodents: II. The variability of phase response curves. J. Comp. Physiol. 106, 253–266.
De Jeu, M.T.G., Hermes, M.L.H.J., Pennartz, C.M.A., 1998. Circadian modulation of membrane properties in slices of rat suprachiasmatic nucleus. NeuroReport 9, 3725–3729.
Goldman, D.E., 1943. Potential, impedance, and rectification in membranes. J. Gen. Physiol. 27, 37–60.
Goldstein, S.A.N., Price, L.A., Rosenthal, D.N., Pausch, M.H., 1996. ORK1, a potassium-selective leak channel with two pore domains cloned from *Drosophila melanogaster* by expression in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 93, 13261–13526.
Hall, A.C., Hoffmaster, R.M., Stern, E.L., Harrington, M.E., Bickar, D., 1997. Suprachiasmatic nucleus neurons are glucose sensitive. J. Biol. Rhythms 12, 388–400.
Hodgkin, A.L., Katz, B., 1949. The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol. 108, 37–77.
Jiang, Z., Nelson, C.S., Allen, C.N., 1995. Melatonin activates an outward current and inhibits I_h in rat suprachiasmatic nucleus neurons. Brain Res. 687, 125–132.
Klein, D.C., Moore, R.Y., Reppert, S.M., 1991. Suprachiasmatic Nucleus: The Mind's Clock. Oxford Press, New York.
Kyrozis, A., Reichling, D.B., 1995. Perforated-patch recording with gramicidin avoids artifactual changes in intracellular chloride concentration. J. Neurosci. Methods 57, 27–35.
Larsen, P.J., Enquist, L.W., Card, J.P., 1998. Characterization of the multisynaptic neuronal control of the rat pineal gland using viral transneuronal tracing. Eur. J. Neurosci. 10, 128–145.
Leonoudakis, D., Gray, A.T., Winegar, B.D., Kindler, C.H., Harada, M., Taylor, D.M., Chavez, R.A., Forsayeth, J.R., Yost, C.S., 1998. An open rectifier potassium channel with two pore domains in tandem cloned from rat cerebellum. J. Neurosci. 18, 868–877.
Lesage, F., Guillemare, E., Fink, M., Duprat, F., Lazdunski, M., Romey, G., Barhanin, J., 1996. TWIK-1, a ubiquitous human weakly inward rectifying K^+ channel with a novel structure. EMBO J. 15, 1004–1011.
Liu, C., Weaver, D.R., Jin, X., Shearman, L.P., Pieschl, R.L., Gribkoff, V.K., Reppert, S.M., 1997. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. Neuron 19, 91–102.
Mason, R., Brooks, A., 1988. The electrophysiological effects of melatonin and a putative melatonin agonist (*N*-acetyltryptamine) on rat suprachiasmatic neurons *in vitro*. Neurosci. Lett. 95, 296–301.
McArthur, A.J., Gilette, M.U., Prosser, R.A., 1991. Melatonin directly resets the rat suprachiasmatic circadian clock *in vitro*. Brain Res. 565, 158–161.
McArthur, A.J., Hunt, A.E., Gillette, M.U., 1997. Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. Endocrinology 138, 627–634.

Nelson, C.S., Marino, J.L., Allen, C.N., 1996. Melatonin receptors activate heterotrimeric G-protein coupled KIR3 channels. NeuroReport 7, 717–720.
Pape, H-C., 1996. Queer current and pacemaker: The hyperpolarization-activated cation current in neurons. Annu. Rev. Physiol. 58, 299–327.
Pennartz, C.M.A., de Jeu, M.T.G., Geurtsen, A.M.S., Sluiter, A.A., Hermes, M.L.H.J., 1998. Electrophysiological and morphological heterogeneity of neurons in slices of rat suprachiasmatic nucleus. J. Physiol. 506, 775–793.
Pusch, M., Neher, E., 1988. Rates of diffusional exchange between small cells and a measuring patch pipette. Pflüg. Arch. 411, 204–211.
Rae, J., Cooper, K., Gates, P., Watsky, M., 1991. Low access resistance perforated patch recordings using amphotericin B. J. Neurosci. Methods 37, 15–26.
Redman, J., Armstrong, S., Ng, K.T., 1983. Free running activity rhythms in the rat: entrainment by melatonin. Science 219, 1089–1091.
Reppert, S.M., Weaver, D.R., Ebisawa, T., 1994. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 13, 1177–1185.
Rudy, B., 1988. Diversity and ubiquity of K channels. Neuroscience 25, 729–749.
Schaap, J., Bos, N.P.A., de Jeu, M.T.G., Geurtsen, A.M.S., Meyer, J.H., Pennartz, C.M.A., 1999. Neurons of the rat suprachiasmatic nucleus show a circadian rhythm in membrane properties that is lost during prolonged whole-cell recording. Brain Res. 815, 154–166.
Shibata, S., Cassone, V.M., Moore, R.Y., 1989. Effects of melatonin on neuronal activity in the rat suprachiasmatic nucleus *in vitro*. Neurosci. Lett. 97, 140–144.
Skinner, D.C., Malpaux, B., 1999. High melatonin concentrations in third ventricular cerebrospinal fluid are not due to galen vein blood recirculation through the choroid plexus. Endocrinology 140, 4399–4405.
Starkey, S.J., Matthew, P.W., Beresford, I.J.M., Hagan, R.M., 1995. Modulation of the rat suprachiasmatic circadian clock by melatonin *in vitro*. NeuroReport 6, 1951–1974.
Stehle, J., Vanecek, J., Vollrath, L., 1989. Effects of melatonin on spontaneous electrical activity of neurons in rat suprachiasmatic nuclei: an *in vitro* iontophoretic study. J. Neural Transm. 78, 173–177.
Strecker, G.J., Wuarin, J.-P., Dudek, E., 1997. $GABA_A$-mediated local synaptic pathways connect neurons in the rat suprachiasmatic nucleus. J. Neurophysiol. 78, 2217–2220.
Sugden, D., McArthur, A.J., Ajpru, S., Duniec, K., Piggens, H.D., 1999. Expression of mt1 melatonin receptor subtype mRNA in the entrained rat suprachiasmatic nucleus: a quantitative RT-PCR study across the diurnal cycle. Mol. Brain Res. 72, 176–182.
van den Top, M., Hermes, M.L.H.J., Delagrange, P., Buijs, R.M., 1998. Melatonin generates an outward potassium current in rat suprachiasmatic nucleus neurons. Soc. Neurosci. Abstr. 24, 696.
Vanecek, J., 1998. Cellular mechanisms of melatonin action. Physiol. Rev. 78, 687–721.
Vanecek, J., Pavlik, A., Illnerova, H., 1987. Hypothalamic melatonin receptor sites revealed by autoradiography. Brain Res. 435, 359–362.
Von Gall, C., Duffield, G.E., Hastings, M.H., Kopp, M.D.A., Dehghani, F., Korf, H.-W., Stehle, J.H., 1998. CREB in the mouse SCN: a molecular interface coding the phase-adjusting stimuli light, glutamate, PACAP, and melatonin for clockwork access. J. Neurosci. 18, 10389–10397.
Wan, Q., Man, H.-Y., Liu, F., Braunton, J., Niznik, H.B., Pang, S.F., Brown, G.M., Wang, Y.T., 1999. Differential modulation of $GABA_A$ receptor function by Mel_{1a} and Mel_{1b} receptors. Nat. Neurosci. 2, 401–403.
Weaver, D.R., Rivkees, S.A., Reppert, S.M., 1989. Localization and characterization of melatonin receptors in rodent brain by *in vitro* autoradiography. J. Neurosci. 9, 2581–2590.
Wickman, K., Clapham, D.E., 1995. Ion channel regulation by G proteins. Physiol. Rev. 75, 865–885.

(*Accepted* 2 *August* 2001)