European Journal of Neuroscience, Vol. 17, pp. 197–204, 2003

The mouse $VPAC_2$ receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide *in vitro*

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Keywords: circadian rhythm, electrophysiology, hypothalamus, neuropeptide

Abstract

Expression of coherent and rhythmic circadian (≈ 24 h) variation of behaviour, metabolism and other physiological processes in mammals is governed by a dominant biological clock located in the hypothalamic suprachiasmatic nuclei (SCN). Photic entrainment of the SCN circadian clock is mediated, in part, by vasoactive intestinal polypeptide (VIP) acting through the $VPAC_2$ receptor. Here we used mice lacking the $VPAC_2$ receptor (*Vipr2*$^{-/-}$) to examine the contribution of this receptor to the electrophysiological actions of VIP on SCN neurons, and to the generation of SCN electrical firing rate rhythms SCN *in vitro*. Compared with wild-type controls, fewer SCN cells from *Vipr2*$^{-/-}$ mice responded to VIP and the $VPAC_2$ receptor-selective agonist Ro 25-1553. By contrast, similar proportions of *Vipr2*$^{-/-}$ and wild-type SCN cells responded to gastrin-releasing peptide, arginine vasopressin or *N*-methyl-D-aspartate. Moreover, VIP-evoked responses from control SCN neurons were attenuated by the selective $VPAC_2$ receptor antagonist PG 99-465. In firing rate rhythm experiments, the midday peak in activity observed in control SCN cells was lost in *Vipr2*$^{-/-}$ mice. The loss of electrical activity rhythm in *Vipr2*$^{-/-}$ mice was mimicked in control SCN slices by chronic treatment with PG 99-465. These results demonstrate that the $VPAC_2$ receptor is necessary for the major part of the electrophysiological actions of VIP on SCN cells *in vitro,* and is of fundamental importance for the rhythmic and coherent expression of circadian rhythms governed by the SCN clock. These findings suggest a novel role of $VPAC_2$ receptor signalling, and of cell-to-cell communication in general, in the maintenance of core clock function in mammals, impacting on the cellular physiology of SCN neurons.

Introduction

The circadian system is driven by output from populations of autonomous single-cell circadian clocks residing in the suprachiasmatic nuclei (SCN) biological pacemaker (Inouye & Shibata, 1994; van Esseveldt *et al*., 2000), which coordinate the activity of multiple oscillators found at other brain and peripheral sites (Yamazaki *et al*., 1998, 2000). In addition to its ability to generate and sustain endogenous rhythmicity, a second key feature of the SCN is the capacity to synchronize (entrain) these rhythms to external photic and nonphotic stimuli.

One neuropeptide that is emerging as an important mediator of photic entrainment is vasoactive intestinal polypeptide (VIP). Cells synthesizing VIP are located in the retinally innervated ventral region of the rodent SCN and give rise to extensive intra- and extra-SCN (hypothalamic and thalamic) connections. Retinal fibres terminate directly on VIP cells (Tanaka *et al*., 1993) and VIP cells express *c-fos* following light stimulation (Romijn *et al*., 1996; Castel *et al*., 1997). Furthermore, the phase-shifting actions of VIP on behavioural rhythms *in vivo* (Piggins *et al*., 1995) and SCN cellular rhythms *in vitro* (Reed *et al*., 2001) resemble the phase-resetting pattern of light on locomotor rhythms. These findings, together with the rhythmic expression of VIP in the SCN under light-dark conditions (Shinohara & Inouye, 1995), provide strong evidence for the involvement of VIP in receiving and encoding light information within the SCN.

The actions of VIP are mediated through a family of G-protein-coupled receptors (Harmar *et al*., 1998), two of which, $VPAC_2$ and PAC_1, are expressed heavily in the rodent SCN (Cagampang *et al*., 1998a, b). Evidence is accumulating to suggest that $VPAC_2$ receptor signalling plays a major role in mediating the effects of VIP and also in maintaining SCN pacemaker function. In rat SCN, the electrophysiological actions of VIP on neurons and on the circadian rhythm of electrical activity are mimicked by the selective $VPAC_2$ receptor agonist Ro 25-1553 (Reed *et al*., 2002). Furthermore, mice overexpressing the human $VPAC_2$ receptor exhibit a shorter free-running period of behavioural activity and re-entrain more quickly to advances in the light–dark cycle, when compared with controls (Shen *et al*., 2000).

Molecular dissection of the SCN pacemaker has revealed the critical role of interlocked positive and negative transcription/translation feedback loops of various 'clock' genes and their protein products. Single-gene mutations of these clock components can lengthen, shorten or abolish completely the period of the endogenous rhythm, reinforcing the view that maintenance of circadian timing has significant genetic components (Young & Kay 2001; Reppert & Weaver

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Received 4 October 2002, revised 29 October 2002, accepted 31 October 2002

doi:10.1046/j.1460-9568.2003.02425.x

2002). The recent demonstration of impaired behavioural and molecular circadian function of mice carrying a null mutation of the $VPAC_2$ receptor (*Vipr2*$^{-/-}$) represents the first illustration that normal clock function is dependent on intercellular signalling through a neuropeptide receptor (Harmar *et al.*, 2002).

Using *Vipr2*$^{-/-}$ mice, we have demonstrated that the electrophysiological actions of VIP on SCN neurons are mediated predominately by the $VPAC_2$ receptor. Furthermore, recordings of wheel-running behaviour and of cellular activity rhythms of SCN cells from *Vipr2*$^{-/-}$ mice clearly show that rhythmic clock function is severely impaired following $VPAC_2$ receptor loss.

Materials and methods

Animals

The University of Manchester colony of *Vipr2*$^{-/-}$ mice was derived from five breeding pairs of homozygous mutant mice provided by The University of Edinburgh [mixed C57BL/6J × 129P2/OlaHsd (129/Ola) background] (Harmar *et al.*, 2002). Additional experiments were conducted on 8th generation *Vipr2*$^{-/-}$ mice (1st generation mice back-crossed into the C57BL/6J background for seven generations; provided by A.J.H). Wild-type (WT) controls included C57BL/6J and 129/Ola mice purchased from Harlan Olac Ltd (Bicester, UK), and C57BL/6J *Vipr2*$^{-/-}$ littermates of 8th generation *Vipr2*$^{-/-}$ mice (provided by A.J.H). Adult male mice were used for all experiments. Animals were maintained on a 12 h light : 12 h darkness cycle [lights on designated as Zeitgeber time (ZT) 0] with free access to food and water. All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Electrophysiological recordings

Detailed descriptions of slice preparation and electrophysiology methodologies have been reported previously (McArthur *et al.*, 2000; Coogan *et al.*, 2001; Reed *et al.*, 2001, 2002). Briefly, adult mice were killed by cervical dislocation and decapitation under halothane anaesthesia, the brain removed and coronal hypothalamic brain slices containing the SCN (400–500 μm thick) prepared with a Vibroslicer. Slices were transferred to and maintained (for up to 48 h) in either a custom–built interface style brain-slice chamber (for recording of cellular activity rhythms) or a PDMI-2 submerged slice microincubator (Medical Systems, Greenvale, NY, USA; for recording drug-evoked responses on cellular activity). Slices were perfused (≈ 1.5 mL/min; 35 °C) with oxygenated (95% O_2/5% CO_2) Earle's balanced salt solution (Sigma) supplemented with 24.6 mM glucose, 26.2 mM sodium hydrogen carbonate and 0.0005% gentamicin. Slices were equilibrated for at least 1 h before electrophysiological recordings commenced.

Single-unit activity of SCN cells was recorded extracellularly using borosilicate glass microelectrodes filled with 2 M NaCl (resistance ≈5 MΩ). Actions potential spikes were amplified (× 20), bandpass filtered (0.3–3 kHz) and discriminated from background using the Neurolog modular system (Digitimer, Welwyn Garden City, UK). The firing rate of each cell was averaged every 5 s and plotted as an integrated firing rate histogram on either a chart recorder or using a PC running Spike 3 software (Cambridge Electronic Design, Cambridge, UK). Cells were recorded from all regions of the SCN.

Drug treatment and analysis

Gastrin-releasing peptide, arginine vasopressin and VIP were purchased from BACHEM (St Helens, UK) whereas *N*-methyl-D-aspartate (NMDA) was from Sigma. Ro 25-1553 and PG 99-465 were kindly supplied from Patrick Robberecht (University of Brussels). All drugs were dissolved in distilled water or DMSO (PG 99-465) to stock solutions, aliquoted and stored at −20 °C. Drugs were diluted in Earle's balanced salt solution to the appropriate concentration on the day of use and bath-applied to the slice through the perfusion line for 5 min. For antagonist studies, PG 99-465 was applied for 10 min prior to and throughout the 5 min VIP application. In firing rate rhythm experiments, PG 99-465 was applied to the slice for 7 h on day 1 *in vitro*, from ZT3 to ZT10.

For assessment of pharmacological agents on the cellular activity of SCN cells, a stable baseline-firing rate was established for at least 5 min before drug treatment. On the basis of previously established criteria (Piggins *et al.*, 1994; Coogan *et al.*, 2001; Reed *et al.*, 2001), a response was considered significant if the neuronal firing frequency during the 5 min drug application was increased (activation) or decreased (suppression) by >20% compared with the baseline firing activity recorded for 5 min immediately preceding drug treatment. Differences in the proportion of cells from each genotype responding to drug application were assessed using the χ^2 test of independence. Differences in response characteristics (e.g. magnitude and duration) between genotypes were determined using unpaired *t*-test whereas VIP-evoked responses in the presence and absence of the antagonist were calculated using paired *t*-tests. Differences were considered significant at $P < 0.05$. Where stated, values are mean ± SEM. Data from commercially available and littermate C57BL/6J controls, for both drug-evoked SCN responsiveness and firing rate rhythm experiments, were identical and grouped into a single data set. Drug-evoked responses from *Vipr2*$^{-/-}$ cells were indistinguishable between 1st and 8th generation mice and were similarly pooled.

For analysis of cellular activity rhythms, the mean spontaneous discharge rate of each cell (measured over the 5 min recording period) and corresponding projected Zeitgeber time were determined. All cells recorded for each genotype were pooled and curve-fitting of the electrical activity rhythm was accomplished using a cubic spline nonparametric regression algorithm (Schluter, 1988). Pooled data for each genotype were analysed to generate a lambda (bandwidth) value, smoothed by a running mean based on the bandwidth estimation (bootstrap replicates, 1000) and plotted as mean ± SEM or mean ± 95% confidence intervals. The time corresponding to the maximum of the smoothed data was taken as the peak in electrical activity.

Behavioural analysis

Six C57BL/6J, 129/Ola and 1st generation *Vipr2*$^{-/-}$ mice were housed individually in cages equipped with a running wheel to monitor circadian rhythms of locomotor activity. Wheel revolutions were detected by a magnetic switch and binned every 10 min. Data acquisition and estimation of free-running periods (during days 3–10 in constant darkness) by χ^2 periodogram was performed using Tau software (Mini-Mitter Co., Sunriver, OR, USA). Mice were maintained initially under a 12 h : 12 h light : darkness cycle (light intensities during the light phase ranged from 40 to 100 lux) then exposed to constant darkness.

Results

$VPAC_2$ receptor loss renders SCN cells less responsive to VIP

To determine if the $VPAC_2$ receptor mediates the electrophysiological effects of VIP on mouse SCN cells, we compared the responsiveness of WT and *Vipr2*$^{-/-}$ SCN neurons to VIP and the selective $VPAC_2$ receptor agonist Ro 25-1553 (Gourlet *et al.*, 1997; both 100 nM). There

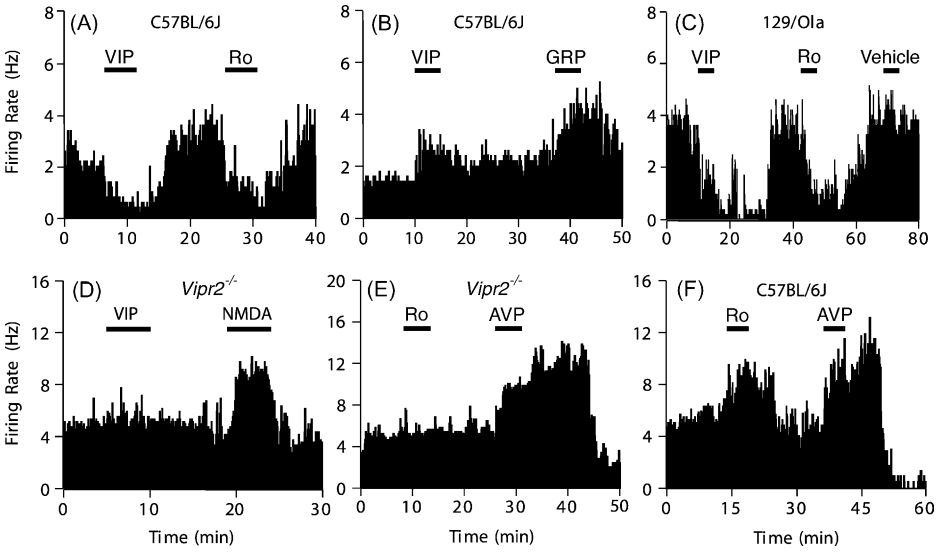

FIG. 1. The electrophysiological actions of VIP in the SCN are lost in VPAC$_2$ receptor-deficient mice. Representative firing rate histograms illustrating the loss of responsiveness of *Vipr2*$^{-/-}$ SCN cells vs. WT controls (C57BL/6J and 129/Ola), to VIP and the selective VPAC$_2$ receptor agonist Ro 25-1553 (Ro), but not to gastrin-releasing peptide (GRP), arginine vasopressin (AVP) or *N*-methyl-D-aspartate (NMDA). Control applications of the perfusion medium (vehicle) did not affect firing rate activity ($n = 16$ out of 17 cells tested). All drugs were tested at 100 nM with the exception of AVP (1 μM) and NMDA (20 μM). Filled bars represent timing and duration of drug application.

was a significant effect of genotype on the responsiveness of SCN cells to VIP and Ro 25-1553 (both $P < 0.01$). Comparable proportions of VIP-responsive cells were seen in both C57BL/6J (53%) and 129/Ola (47%) WT controls (Fig. 1A–C). In contrast, only 14% of *Vipr2*$^{-/-}$ cells responded to VIP, whereas 68/79 (86%) cells failed to respond (Fig. 1D). Both activation and suppression responses were found for all genotypes (Fig. 2). The profile of VIP-evoked activations (magnitude $151 \pm 84\%$ of control baseline firing rate; duration 13.5 ± 4.4 min; $n = 6$) and suppressions (magnitude $80 \pm 5\%$; duration 13.1 ± 4.8 min; $n = 5$) from *Vipr2*$^{-/-}$ SCN cells did not differ significantly from activations (magnitude $86 \pm 17\%$; duration 11.4 ± 1.4 min; $n = 18$) or suppressions (magnitude $81 \pm 5\%$; duration 17.4 ± 3.1 min; $n = 17$) evoked from pooled WT SCN neurons (all $P > 0.05$).

The VPAC$_2$ receptor agonist Ro 25-1553 was equally effective as VIP at evoking responses from C57BL/6J (46%) and 129/Ola (50%) WT cells (Fig. 1A, C and F) suggesting that the actions of VIP are mediated predominately via the VPAC$_2$ receptor. The dramatic reduction in cellular responsiveness to Ro 25-1553 in *Vipr2*$^{-/-}$ mice (3%) vs. WT controls supports this contention (Figs 1E and 2). To investigate further the necessity of the VPAC$_2$ receptor for the actions of VIP in the SCN, we assessed in C57BL/6J mice the effect of VIP in the presence and absence of the selective VPAC$_2$ receptor antagonist PG 99-465 (Moreno *et al.*, 2000). In five cells suppressed by VIP, PG 99-465 antagonized both the magnitude (VIP alone, $-93 \pm 3\%$; VIP + PG 99-465, $-23 \pm 15\%$; $P < 0.01$) and duration (VIP alone, 17.3 ± 2.2 min; VIP + PG 99-465, 0.9 ± 0.6 min; $P < 0.01$) of the VIP response (Fig. 3A). PG 99-465 partially antagonized the VIP-evoked activation in two cells with respect to both response magnitude (VIP alone, 98%; VIP + PG 99-465, 71%) and duration (VIP alone, 12.8 min;

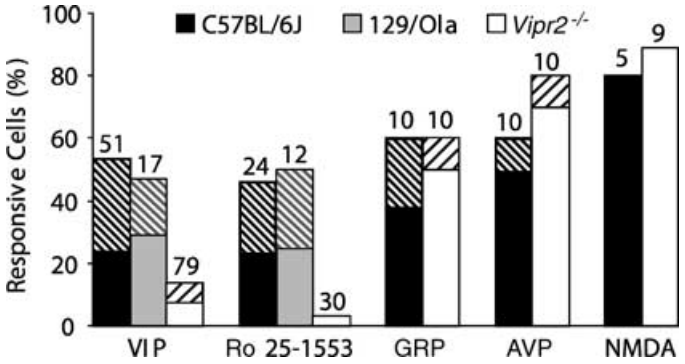

FIG. 2. Summary of responsiveness of WT (pooled C57BL/6J and 129/Ola data) and *Vipr2*$^{-/-}$ SCN cells to VIP, Ro 25-1553, gastrin-releasing peptide (GRP), arginine vasopressin (AVP) or *N*-methyl-D-aspartate (NMDA). All drugs were tested at 100 nM with the exception of AVP (1 μM) and NMDA (20 μM). The solid and hatched portion of each bar indicates the proportion of cells activated or suppressed, respectively, by each drug. The values above each bar represent number of cells tested.

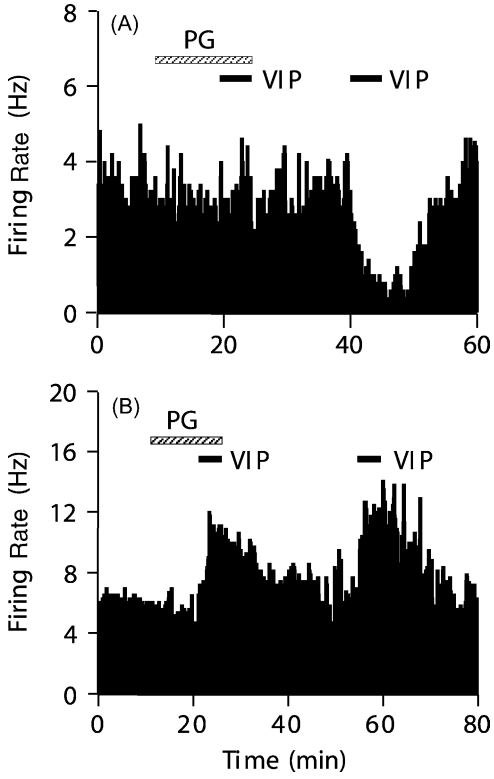

FIG. 3. The electrophysiological actions of VIP in the mouse SCN are antagonized by VPAC$_2$ receptor blockade. Representative firing rate histograms of C57BL/6J SCN cells depicting the sensitivity of (A) VIP-evoked suppression and (B) activation responses to the selective VPAC$_2$ receptor antagonist PG 99-465 (PG). Both VIP and PG 99-465 were used at 100 nM. The solid and hatched bars represent the timing and duration of VIP and PG 99-465 application, respectively.

VIP + PG 99-465, 9.2 min; Fig. 3B). Application of PG 99-465 alone had no effect on SCN cellular activity ($n = 14$ out of 15 cells).

To ensure that the reduced responsiveness of SCN cells to VIP and Ro 25-1553 in *Vipr2*$^{-/-}$ mice was not attributable to a generalized loss of G-protein receptor function, C57BL/6J and *Vipr2*$^{-/-}$ mice were tested with gastrin-releasing peptide and arginine vasopressin, two neuropeptides that act through G-protein-coupled receptors and potently affect SCN electrical activity (Liou & Albers, 1989; Tang & Pan, 1993; Piggins *et al*., 1994; Ingram *et al*., 1998). Comparable numbers of SCN cells responded to gastrin-releasing peptide and arginine vasopressin in both genotypes (Figs 1B, E, F and 2). Cells from WT and *Vipr2*$^{-/-}$ mice were also equally responsive to ionotropic glutamate receptor activation by NMDA (Figs 1D and 2).

The VPAC$_2$ receptor is necessary for behavioural and SCN cellular rhythmicity

Previous wheel-running analysis of VPAC$_2$ receptor null mice showed that this mutation results in severe disturbances of the circadian timing system with *Vipr2*$^{-/-}$ mice exhibiting lower activity levels, altered patterns of phase resetting following light-dark cycle shifts and immediate disruption of rhythmicity on entering constant conditions (Harmar *et al*., 2002). A similar behavioural phenotype was observed for our colony of *Vipr2*$^{-/-}$ mice. Wheel-running behaviour of C57BL/6J and 129/Ola WT mice, and *Vipr2*$^{-/-}$mice, showed good entrainment to a 12 h light : 12 h darkness lighting regimen (Fig. 4). When transferred to constant darkness, WT mice maintained robust circadian rhythms of activity with near 24 h periodicity (C57BL/6J 23.8 ± 0.1 h; 129/Ola 23.7 ± 0.1 h). In contrast, the behaviour of *Vipr2*$^{-/-}$ mice appeared arrhythmic with no clear pattern of activity onset and offset, indicative of impaired clock function.

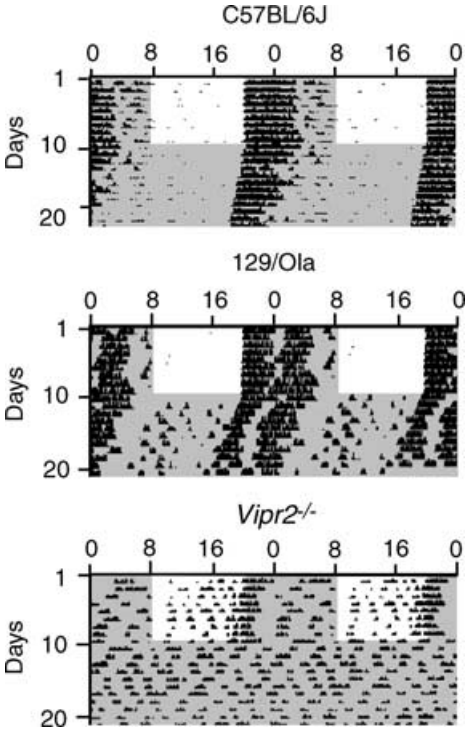

FIG. 4. The mouse VPAC$_2$ receptor is necessary for the expression of a circadian rhythm of behavioural activity under constant conditions *in vivo*. Representative wheel-running profiles of C57BL/6J, 129/Ola and *Vipr2*$^{-/-}$ mice maintained under a 12 h : 12 h light : darkness cycle (time of lights-on 07.00 h; days 1–9) or in constant darkness (days 10–21). Behavioural activity in WT and *Vipr2*$^{-/-}$ mice showed entrainment under light : dark conditions, but coherent rhythms of wheel-running persisted only in WT mice while under conditions of constant dark. Shaded areas represent periods of darkness. Wheel-running actograms are double-plotted, with the last 24 h of each line repeated on the next line, to aid visual inspections of behavioural rhythms.

A critical feature of the SCN is that expression and cycle length of the clock is conserved faithfully *in vitro* with rhythms of neuronal activity matching the behavioural phenotype of the animal, as has been demonstrated for *Clock* (Herzog *et al*., 1998) and *mCry1/mCry2* (Albus *et al*., 2002) mutant mice, and *tau* mutant hamsters (Davies & Mason, 1994; Liu *et al*., 1997). To determine if the loss of behavioural rhythmicity of *Vipr2*$^{-/-}$ mice *in vivo* is matched by a loss of electrical activity rhythms *in vitro*, we next examined the firing rate rhythm of SCN cells recorded from WT and *Vipr2*$^{-/-}$mice. SCN cells from C57BL/6J and 129/Ola WT mice expressed the expected circadian rhythm of electrical activity with robust peaks in firing rate during the middle of the light phase on both day 1 and day 2 *in vitro* (Fig. 5A and B). The electrical activity rhythm in all WT slices peaked during the projected day and slowed to low frequencies during the projected night. The calculated period of the *in vitro* rhythm closely resembled the period of behavioural activity for each genotype (C57BL/6J 23.88 h; 129/Ola 23.77 h). Analysis of pooled data from all WT controls showed peaks in firing of 4.1 ± 0.3 Hz at ZT6.7 (day 1) and 3.8 ± 0.3 Hz at ZT6.6 (day 2; $n = 22$ slices, 1631 cells).

In contrast, no circadian rhythm in firing rate was observed for either 1st or 8th generation *Vipr2*$^{-/-}$ mice on either day 1 or day 2 *in vitro* (Fig. 5C). No overt peaks in the electrical activity rhythm were found during projected day or projected night phases in any individual *Vipr2*$^{-/-}$ SCN slice. Pooled results from both generations of *Vipr2*$^{-/-}$mice ($n = 18$ slices, 1317 cells) indicated that these SCN cells did not exhibit large increases in firing rate vs. the pooled data for WT controls (Fig. 5D). Furthermore, the 95% confidence intervals of the smoothed firing rate data from all WT and *Vipr2*$^{-/-}$ cells showed that the median frequency of *Vipr2*$^{-/-}$cells was significantly lower than that of WTs at virtually all recording phases, overlapping at only a few late night time points. This pattern of persistently low neuronal activity of *Vipr2*$^{-/-}$ cells at any phase *in vitro* is unlike that reported for another behaviourally arrhythmic transgenic mouse (*mCry1*$^{-/-}$/*mCry2*$^{-/-}$), which shows normal levels of spontaneous activity during the first 24 h cycle *in vitro* before damping to low levels in subsequent cycles (Albus *et al*., 2002). It is also different to the electrophysiological activity of SCN cells from the Brattleboro rat which, although lacking a prominent physiological clock output (clock gene-driven arginine vasopressin secretion), sustains day–night variability in SCN neuronal firing (Ingram *et al*., 1996). Therefore, the absence of rhythmic SCN electrical activity in the *Vipr2*$^{-/-}$ mice is novel and distinct from that described previously in other SCN clock mutant animals.

Frequency distribution analysis of all WT and *Vipr2*$^{-/-}$ data revealed that the activity of SCN cells from *Vipr2*$^{-/-}$ mice was significantly lower in amplitude than WT controls during both the light and dark phases (both $P < 0.0001$, Kruskal–Wallis one-way ANOVA; Fig. 5E). Furthermore, when classified into 2 Hz bins (0–1.99, 2.00–3.99, 4.00–5.99, >6.0 spikes/s), clear differences in the distribution of spontaneous firing rates between WT and *Vipr2*$^{-/-}$ mouse SCN neurons were apparent. Over 80% of *Vipr2*$^{-/-}$ SCN cells fired at less than 2 Hz compared with 48% of WTs. Moreover, only 4% of *Vipr2*$^{-/-}$ mouse SCN neurons discharged at >4 Hz compared with 20% of WT SCN cells. These findings demonstrate clearly that the behavioural and molecular deficits incurred by VPAC$_2$ receptor loss are accompanied by dramatic changes in SCN electrical activity.

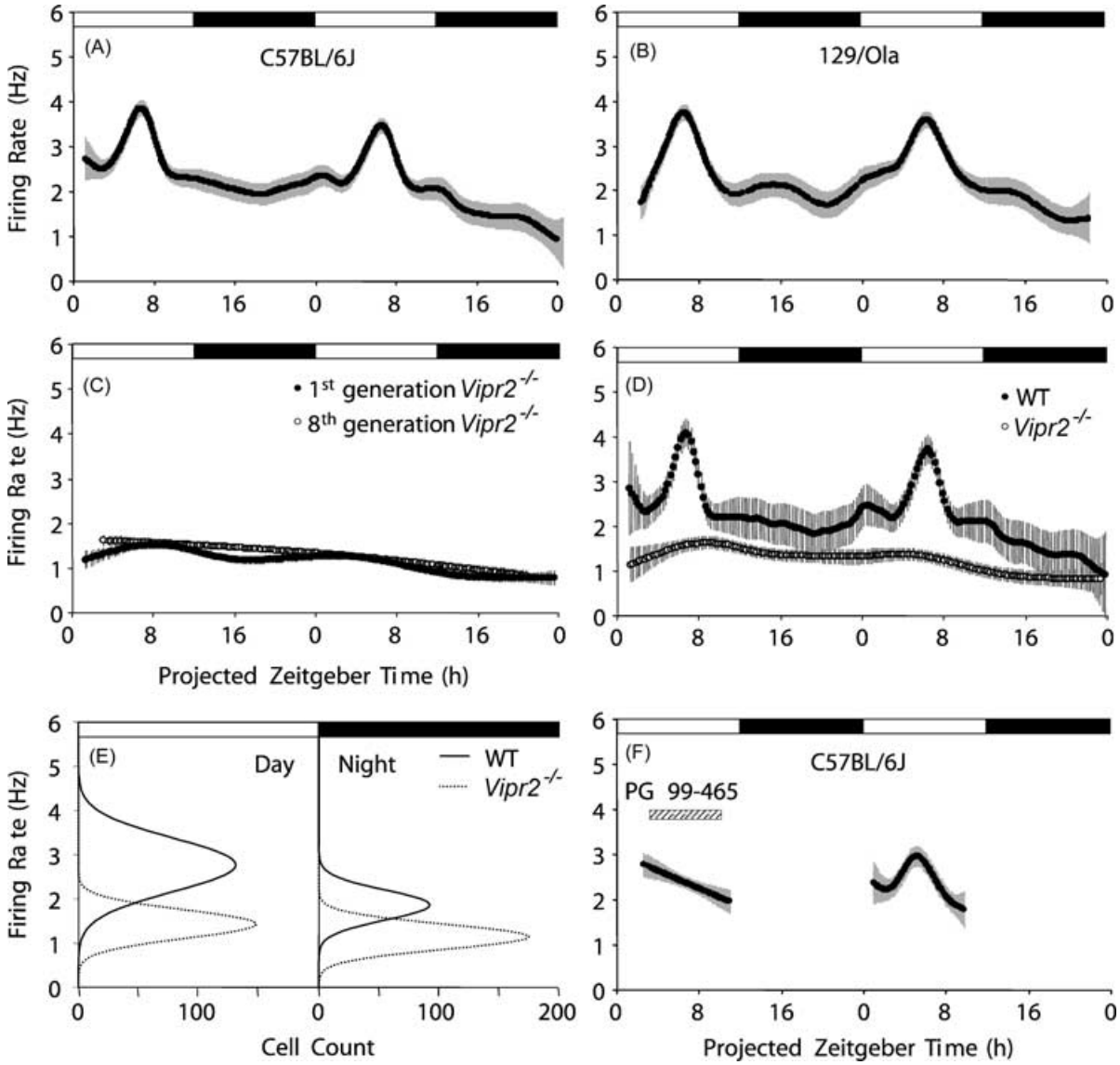

FIG. 5. The mouse VPAC$_2$ receptor is necessary for the expression of a circadian rhythm of ensemble SCN neuronal activity *in vitro*. Firing rate rhythm of electrical activity of (A) C57BL/6J ($n = 14$ slices, 1053 cells) and (B) 129/Ola ($n = 8$ slices, 578 cells) WT controls. (C) Loss of electrical activity rhythm in both 1st ($n = 13$ slices, 745 cells; solid circles) and 8th ($n = 5$ slices, 572 cells; open circles) generation *Vipr2*$^{-/-}$ mice. (D) WT control data (closed circles) represent pooled results from C57BL/6J (both commercially available and congenic littermate controls) and 129/Ola mice, while data for *Vipr2*$^{-/-}$ mice (open circles) are pooled results from 1st and 8th generation mutants. (E) Density plots showing the frequency distribution of the firing rates of all cells during the light or dark phases are based on the smoothed data presented graphically in panel (D). Light-phase cell counts: WT 1172, *Vipr2*$^{-/-}$ 696; dark phase cell counts: WT 459, *Vipr2*$^{-/-}$ 621. (F) The selective VPAC$_2$ receptor antagonist PG 99-465 (10 nM, 7 h application from ZT3–10) abolished the expected rhythm of SCN neuronal firing in C57BL/6J slices on day 1 *in vitro*, which recovered on day 2 *in vitro*. Data from individual experiments for each genotype were pooled, smoothed and plotted as mean ± SEM (A–C and F) or mean ± 95% confidence intervals (D). Open and filled bars represent periods of light and darkness, respectively. Hatched bar shows timing and duration of PG 99-465 administration.

To test the possibility that loss of rhythmic SCN function *in vitro* was attributable mainly to developmental abnormalities associated with loss of the VPAC$_2$ receptor, we investigated the effect of VPAC$_2$ receptor blockade on expression of the circadian rhythm in firing rate in C57BL/6J mice. Application of PG 99-465 on day 1 *in vitro* (7 h, from ZT3–10) blocked the anticipated midday peak in activity in all slices tested ($n = 3$; Fig. 5F). The rhythm in neuronal firing was restored on day 2 although the peak in firing rate was blunted (≈ 2.7 Hz) and phase advanced by ≈ 1.3 h (peak at $\approx$ZT5.0) with respect to untreated slices (peak of ≈ 3.6 Hz at $\approx$ZT6.3). Moreover, the absence of rhythmic activity on day 1 *in vitro* was not observed in any untreated WT slice ($n = 10$ out of 10).

Discussion

The VPAC$_2$ receptor plays an essential role in the mouse SCN for the coordination of circadian rhythms of wheel-running and of core clock gene expression (Harmar *et al*., 2002). Our present results now clearly demonstrate that the mouse VPAC$_2$ receptor is necessary for the generation of a circadian rhythm of SCN neuronal activity, reflected in the SCN at the level of single-unit firing rate. The loss of *in vitro* electrical activity rhythms in *Vipr2*$^{-/-}$ mice was replicated by chronic (7 h) administration of the VPAC$_2$ receptor antagonist PG 99-465; this effect appeared to be a cumulative consequence of pharmacological VPAC$_2$ receptor blockade as short-term (10–15 min) application of

PG 99-465 did not alter SCN cellular activity. SCN cellular activity in WT slices treated for 7 h with PG 99-465 did not however, drop to the very low levels seen in *Vipr2*$^{-/-}$ SCN slices. This suggests that either longer exposure to this $VPAC_2$ receptor antagonist is necessary to achieve this level of suppression or that other as yet uncharacterized mechanisms compromise cellular activity in *Vipr2*$^{-/-}$ SCN neurons. The absence of high firing (> 4 Hz) cells in *Vipr2*$^{-/-}$ mice at any day or night phase indicates that the mutation results in fundamental disruption of clock cell physiology. However, *Vipr2*$^{-/-}$ cells retain the capacity to express high firing rates (8–12 Hz), as noted following stimulation with NMDA, gastrin-releasing peptide or arginine vasopressin. Importantly, we show for the first time that WT mouse SCN cells respond to endogenous neuropeptides (VIP, gastrin-releasing peptide, arginine vasopressin) similarly to SCN neurons recorded from other species, e.g. rat and hamster (Liou & Albers, 1989; Tang & Pan, 1993; Piggins *et al*., 1994; Ingram *et al*., 1996, 1998).

Because SCN cells from *Vipr2*$^{-/-}$ mice are still capable of expressing electrical activity, it appears that $VPAC_2$ receptor activation is either a critical component in rhythm generation processes and/or coordinates coherent phasic output from this structure. The observations of poor electrical and locomotor wheel-running rhythms seen in *Vipr2*$^{-/-}$ mice might not be sufficient to resolve the role of the $VPAC_2$ receptor, as both are measures of clock output. Although long-term monitoring of individual SCN cellular activity could clarify the possibility that single cells remain rhythmic, inconsistencies have been reported with these methods, for example the assessment of rhythmic SCN electrical activity in *Clock* mutant mice (compare Herzog *et al*., 1998 with Nakamura *et al*., 2002). However, the importance of electrical output should not be understated as recent evidence from the *Drosophila* clock suggests that neuronal activity also acts as a feedback mechanism to drive the cycling of molecular (clock gene) rhythms in pacemaker cells (Nitabach *et al*., 2002). The observation that individual SCN *Vipr2*$^{-/-}$ cells do not retain rhythmic clock gene expression (Harmar *et al*., 2002), together with our cellular and behavioural findings, favour the notion that the $VPAC_2$ receptor is of critical importance for circadian pacemaker function, rather than coupling output processes to a still-oscillating SCN clock.

The abnormal phenotype of *Vipr2*$^{-/-}$ mice is most likely to be a consequence of altered signalling through the $VPAC_2$ receptor by VIP. In WT mice, the proportion of SCN cells that responded to VIP or the $VPAC_2$ receptor agonist Ro 25-1553 were identical, whereas $VPAC_2$ receptor blockade attenuated VIP-evoked suppressions, and to a lesser extent activations, in all cells tested. The severe loss of responsiveness of *Vipr2*$^{-/-}$ SCN cells to VIP or Ro 25-1553 strengthens the contention that the actions of VIP in the mouse SCN, at least at the cellular level, are mediated predominately via the $VPAC_2$ receptor. A similar finding has been reported for rat SCN (Reed *et al*., 2002), a species in which the phase-shifting effects of VIP *in vitro* are mimicked by $VPAC_2$ receptor ligands, but not by agents selective for other VIP receptors (Reed *et al*., 2001). Further behavioural analysis is necessary to determine whether the pivotal role the $VPAC_2$ receptor plays *in vitro* is also realized for the phase-dependent actions of VIP in the intact animal (Albers *et al*., 1991; Piggins *et al*., 1995).

The small proportion of *Vipr2*$^{-/-}$ SCN cells that still responded to VIP, but not to Ro 25-1553, raises the possibility that another receptor contributes to the actions of VIP in this structure. The PAC_1 receptor is the most likely candidate as, like the $VPAC_2$ receptor, this receptor is densely expressed in the rodent SCN (Usdin *et al*., 1994; Hannibal *et al*., 1997; Vertongen *et al*., 1997; Cagampang *et al*., 1998a, b; Shinohara *et al*., 1999). The finding that the PAC_1 receptor mediates some of the electrophysiological actions of VIP in rat SCN supports this contention (Reed *et al*., 2001). These effects could be attributed to the PAC_1*short* splice variant of this receptor, which is found in the SCN (Ajpru *et al*., 2002; Shinohara *et al*., 2002) and binds VIP with an uncharacteristically high affinity (Dautzenberg *et al*., 1999). The PAC_1 receptor is functional in mouse SCN where its putative role is to underlie the actions of pituitary adenylate cyclase-activating polypeptide on the circadian clock (Chen *et al*., 1999; Harrington *et al*., 1999; Hannibal *et al*., 2001), a peptide colocalized with glutamate in the direct retinal projection to the SCN (Hannibal *et al*., 2000). However, even if activation of the PAC_1 receptor contributes to the responsiveness of the SCN to VIP, findings from *Vipr2*$^{-/-}$ mice show that the presence of only PAC_1 in the SCN is evidently not sufficient to impose temporal organization on molecular, cellular or behavioural rhythms. Consistent with this idea, loss of PAC_1 receptor function in PAC_1 receptor-deficient mice only subtly impacts on normal circadian processes; these mice remain rhythmic but show altered behavioural phase shifts and clock gene activation in response to light (Hannibal *et al*., 2001).

Rhythmicity of the SCN circadian pacemaker is synchronized by external photic stimulation and VIP acting via the $VPAC_2$ receptor is implicated strongly in this process (Tanaka *et al*., 1993; Inouye & Shibata, 1994; Piggins *et al*., 1995; Shinohara & Inouye, 1995; Romijn *et al*., 1996; Shen *et al*., 2000; Reed *et al*., 2001). The recent demonstration that VIP induces SCN *per1* and *per2* clock gene expression at late night phases shows that VIP neurotransmission can invoke the molecular machinery that underpins light resetting of SCN clock function (Nielsen *et al*., 2002). The inability of *Vipr2*$^{-/-}$ mice to sustain coherent circadian rhythms of clock gene expression, cellular activity and behaviour reported here and elsewhere (Harmar *et al*., 2002) reveals a more fundamental role of $VPAC_2$ receptor signalling in core clock function. The findings that altered SCN VIP content, distribution or projections are correlated with a diminution of circadian function (Scarbrough *et al*., 1996; Sollars & Pickard, 1996; Shima *et al*., 2000; Duncan *et al*., 2001), lends further support to the idea that the VIP–$VPAC_2$ receptor system is necessary for imposing temporal organization to SCN clock processes.

How disrupted intercellular communication following $VPAC_2$ receptor loss leads to arrhythmic clock function remains to be determined, although it has been suggested that rhythmic release of VIP serves as a paracrine reinforcing signal to maintain cell-to-cell coupling in the SCN (Harmar *et al*., 2002). Alternatively, it is possible in *Vipr2*$^{-/-}$ mice that weakened cellular coupling results from a shortened or lengthened circadian period of (rhythmic) individual SCN cells, as hypothesized for *Clock* mutant mice (Nakamura *et al*., 2002). However, our observation that only a very small proportion (4%; 56 of 1317 neurons recorded) of *Vipr2*$^{-/-}$ SCN cells are spontaneously active above 4 Hz suggests either that considerably few *Vipr2*$^{-/-}$ SCN neurons are rhythmic or that oscillations of SCN cellular activity remain below the level of detection. In either case, the electrical output of *Vipr2*$^{-/-}$ SCN cells is insufficient to drive overt behavioural rhythms. Disruption of cell-to-cell communication and absence of rhythmic clock function in *Vipr2*$^{-/-}$mice appears to be attributable directly to $VPAC_2$ receptor loss, rather than to an indirect developmental defect, based on a number of pieces of evidence. In the present study, a selective $VPAC_2$ receptor antagonist blocked the actions of VIP on WT SCN cells, thus mimicking the loss of cellular responsiveness to VIP and Ro 25-1553 seen in the SCN of *Vipr2*$^{-/-}$ mice. Moreover, long-term treatment with this antagonist ablated the daytime peak in electrical activity of WT SCN neurons, which resembles the absence of a cellular activity rhythm in *Vipr2*$^{-/-}$ mice. Finally, *Vipr2*$^{-/-}$ mice show normal retinal innervation of the SCN and typical distributions of VIP, substance P, neuropeptide Y, serotonin and galanin (levels of arginine vasopressin are, however, slightly depressed in

Vipr2$^{-/-}$ mice – consistent with impaired output from the clock), results which further refute the possibility that a gross developmental abnormality underlies the phenotype of *Vipr2*$^{-/-}$ mice (Harmar *et al*., 2002; D.J.C & H.D.P, unpublished observations).

The results presented here demonstrate that the behavioural and molecular deficits attributable to $VPAC_2$ receptor loss are reflected in fundamental abnormalities of cellular physiology within the core SCN clock *in vitro*. Moreover, we also show that the $VPAC_2$ receptor is the predominant receptor for mediating the electrophysiological actions of VIP on mouse SCN cells, further establishing the essential role of $VPAC_2$ receptor signalling in VIP-mediated alterations of SCN clock function. Taken in sum, these findings demonstrate the importance of a functional neuropeptide receptor complex in physiological rhythms of SCN clock cells.

Acknowledgements

We wish to thank Richard Preziosi for assistance with the cubic spline statistical analysis, Andrew Loudon for critical review of the manuscript, Patrick Robberecht for the generous gifts of Ro 25-1553 and PG 99-465, and Alert Meyer-Spasche for technical assistance. This work was supported by the BBSRC and MRC.

Abbreviations

NMDA, *N*-methyl-D-aspartate; SCN, suprachiasmatic nuclei; VIP, vasoactive intestinal polypeptide; WT, wild-type.

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