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BRAIN RESEARCH

Short Communication

Lack of the PAC_1 receptor alters the circadian expression of VIP mRNA in the suprachiasmatic nucleus of mice

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ABSTRACT

PACAP in the retinohypothalamic tract mediates photic information to the suprachiasmatic nucleus via the PAC_1 receptor. The diurnal and circadian VIP mRNA expressions in the suprachiasmatic nucleus of PAC_1–/– and wild type mice were quantified. During light/dark cycles identical VIP mRNA rhythms were found while the oscillation pattern differed between the two types of animals during constant darkness. The results show that the circadian VIP mRNA expression is influenced by the absence of PAC_1 signalling.

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In mammals, the master circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus generates biological rhythms of physiology and behaviour with periods of near-24-h (circadian rhythms). The SCN is composed of oscillatory cells in which the rhythmic circadian activity is dependent on cyclic expression of 'clock genes' (Lowrey and Takahashi, 2004; Reppert and Weaver, 2002).

The neuropeptide vasoactive intestinal polypeptide (VIP) is expressed in the neurons of the ventrolateral part of the SCN and VIPergic neurons project to cells within the entire SCN as well as to cells of other brain areas (Abrahamson and Moore, 2001; Moore et al., 2002). Accumulating evidence from studies using exogenous application of VIP and experiments with mice deficient in VIP or the VIP receptor $VPAC_2$ suggests that VIP plays an essential role in both re-setting to light and in maintenance of ongoing rhythmicity. VIPergic signalling in SCN thus seems involved both in maintaining the molecular timekeeping within individual neurons and the cell-to-cell synchronisation (Aton et al., 2005; Colwell et al., 2003; Cutler et al., 2003; Harmar et al., 2002; Hughes et al., 2004; Maywood et al., 2006; Reed et al., 2001; Watanabe et al., 2000). The circadian pacemaker in the SCN is daily adjusted to environmental light and darkness, but the mechanism by which external light modifies the activity of the SCN neurons is still incompletely understood (Aton and Herzog, 2005). The VIPergic neurons in the SCN receive a direct retinal input via the retinohypothalamic tract (RHT) originating from a subset of intrinsically photosensitive retinal ganglion cells (ipRGC) (Berson, 2003). These ganglion cells express the classical neurotransmitter glutamate and the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) (Hannibal et al., 2000), in addition to the photopigment melanopsin responsible for the non-image forming light input regulating the circadian

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Abbreviations: CT, circadian time; DD, Constant darkness; ipRGC, Intrinsic photosensitive retinal ganglion cells; LD, Light/dark; PACAP, Pituitary adenylate cyclase activating polypeptide; PAC_1, PACAP receptor type 1; RHT, Retinohypothalamic tract; RT-PCR, Reverse transcriptase polymerase chain reaction; SCN, Suprachiasmatic nucleus; VIP, Vasoactive intestinal polypeptide; $VPAC_1$, VIP receptor type 1; $VPAC_2$, VIP receptor type 2; WT, Wild type; ZT, zeitgeber time

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system (Berson et al., 2002; Gooley et al., 2001; Hannibal et al., 2002; Hattar et al., 2002; Provencio et al., 2002).

Glutamate has for years been considered an important mediator of adjustment in circadian timing in response to light (Ebling, 1996). The function of PACAP as transmitter in RHT and its interplay with glutamate has been unravelled more recently by in vitro studies, studies using PACAP injection into the SCN or its vicinity, PACAP receptor blockade, and mutant mice lacking PACAP or the PACAP receptor type 1 (PAC_1) (Bergström et al., 2003; Chen et al., 1999; Hannibal et al., 1997, 2001; Harrington et al., 1999). PACAP seems to be required for normal light-induced resetting of the circadian system and plays a role in both light-induced phase advance and phase delay. In addition, PACAP modulates glutamate induced phase shifts and has a phase advancing effect at day (Fahrenkrug, 2006; Hannibal, 2006). PACAP can exert its actions via three different G-protein coupled receptors. The PAC_1 is specific for PACAP while the VIP receptor type 1 ($VPAC_1$) and $VPAC_2$ are shared with VIP (Harmar et al., 1998). mRNA encoding the PAC_1 receptor is expressed throughout the SCN but especially in the vetrolateral VIP containing cells (Kalamatianos et al., 2004).

Day/night fluctuations of the VIP expression in SCN has been shown but with considerable interspecies differences. In the rat, VIP and its mRNA have been shown to exhibit daily rhythms with the highest levels during the dark (Albers et al., 1990; Okamoto et al., 1991; Takahashi et al., 1989; Yang et al., 1993; Zoeller et al., 1991), while no oscillation of VIP nor its mRNA was seen during constant darkness (DD) (Shinohara et al., 1993; Takeuchi et al., 1992). Most likely the diurnal variation of VIP in rats depends on opposing effects of light and darkness as light has been shown to decrease while darkness to increase VIP in the SCN (Albers et al., 1987; Shinohara et al., 1999). In the Syrian hamster, no oscillation of VIP mRNA was found during light/dark (LD) (Duncan et al., 2001; Lucas et al., 1998). In contrast to these findings, VIP has shown to be rhythmically expressed in the mouse SCN both during LD cycles and DD (Dardente et al., 2004). In mice SCN, it thus seems that the circadian pacemaker determines the VIP expression.

In a previous study, we reported that PACAP is able to provoke a marked induction of VIP gene expression in neuronal cells (Georg and Fahrenkrug, 2000). In order to elucidate the role of PAC_1 receptor signalling on the temporal profiles of VIP in the SCN, we quantified the changes in VIP mRNA by real-time reverse transcriptase (RT)-PCR in SCN of mice lacking the PAC_1 receptor (PAC_1–/–) and their wild type (WT) littermates during a 12:12 h LD cycle as well as in 48 h of DD.

A total of 96 male and 96 female WT and PAC_1–/– mice (3–8 months old) from a F1–F4 strain of 129/Sv mice were used in this study. The animals were maintained with food and water ad libitum in a 12:12 h LD cycle for at least 2 weeks before the experiment. 48 animals of each genotype were killed by decapitation at the following ZT times: 4, 8, 12, 16, 20 and 24, where ZT designated Zeitgeber, ZT 0 corresponds to lights ON and ZT 12 corresponds to light OFF. Eight animals, of both sexes, were included at each time point. Another 48 animals of each genotype were killed by decapitation during the second cycle of DD (designated circadian time: CT 4, CT 8, CT 12, CT 16, CT 20, CT 24). Eight animals of both sexes were included at each time point. Decapitation during the dark period was performed in dim red light (<5 lx). After decapitation the brains were rapidly removed, frozen on dry ice and kept at −80 °C until further processing. Hypothalamic coronal slices (300 μm) containing the SCN were cut in a cryostat and the entire SCN dissected as previously described (Fahrenkrug et al., 2005). All animal experiments were performed in accordance with the law on animal experiments in Denmark (publication No. 382, June 10th 1987).

Total RNA from SCN of individual animals and cDNA were made as described previously (Hannibal et al., 2005). Mouse cortex cerebri RNA was used to make a large batch of cDNA used for standard curves. Five serial five-fold dilutions were made and frozen in aliquots. The most concentrated sample held: cDNA from 50 ng total RNA/μl and the least concentrated: cDNA from 80 pg total RNA/μl. Doublets of 2.5 μl of each standard were assayed on each plate; the highest standard was arbitrarily set to 12,500 and the lowest to 20.

Real-time PCR was performed in 25 μl reactions containing cDNA from 25 ng total RNA and using TaqMan Universal PCR Master Mix containing AmpErase7UNG (Applied Biosystems). For VIP expression Mm00660234_m1 Assay-on-Demand (Applied Biosystems) was used. The primers and TaqMan probe for the β2-microglobulin (β2MG) assay used as internal control were designed using Primer Express software (Applied Biosystems). 200 nM probe (VIC-CCTCAAATTCAAGTATACTCACGCCACCCA-TAMRA) and 600 nM of both the forward: CGGCTTGTATGCTATCCA-GAAAA and reverse primer AGTATGTTCGGCTTCCCATTCTC were used. It was verified that neither assay detected DNA in an amount equal to the amount of RNA added to the reactions. The amount of β2MG mRNA was not found to vary as a function of time in any of the groups of animals. The VIP and the β2MG assays were run in separate wells on the same plate and all samples, standards, and the non-template negative controls were made in duplicates. The ABI prism 7000 SDS software program (Applied Biosystems) was used to calculate the concentrations (in arbitrary units) of VIP and β2MG mRNA. The amount of VIP mRNA was normalised with the amount of β2MG mRNA obtained from the same run leading to a normalised VIP concentrations. No differences in neither VIP nor β2MG mRNA expression was found between the genders in neither WT nor PAC1–/– animals.

Levels of normalised VIP mRNA were presented as means ± S.E.M. Diurnal and circadian changes in mRNA were analysed using the method for cosinor-rhythmometry as described by Nelson et al., with the period set to 24 h (Nelson et al., 1979). The model fit was then tested using the GLM procedure in the SAS statistical software package of normalised VIP mRNA (1994), $P<0.05$ was considered statistically significant.

During the 12 h/12 h LD cycle, VIP mRNA levels in the SCN determined by real-time RT-PCR displayed rhythmic oscillations as a function of a 24 h cycle in both WT and PAC_1–/– mice (Fig. 1). PAC_1–/– mice are also able to entrain to 12 h/12 h LD (Hannibal et al., 2001) and the phases of the 24 h VIP oscillations were identical in both PAC1–/– and WT mice. The calculated maximal VIP mRNA expression was found immediately before midday (WT: ZT 5.2 and PAC_1–/–:

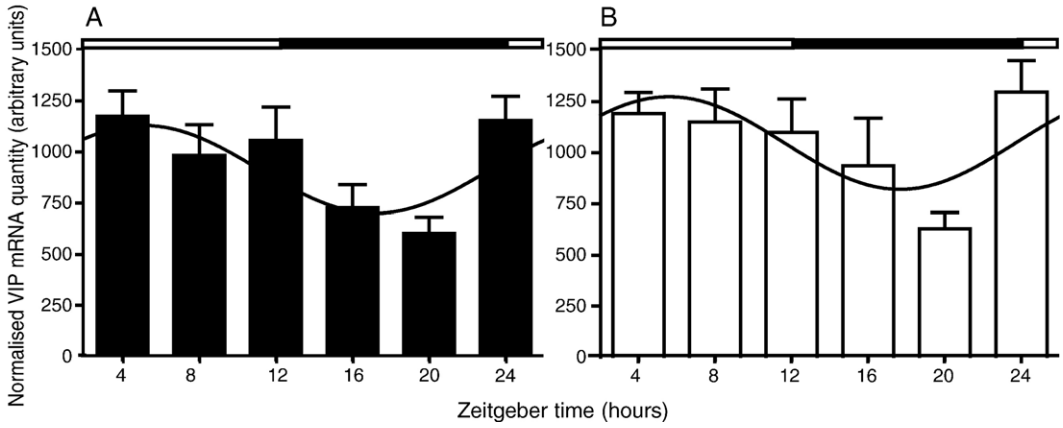

Fig. 1 – Diurnal expression of VIP mRNA in SCN from wild type (A) and PAC_1–/– (B) mice. VIP mRNA concentration in SCN of mice sacrificed every 4th hour during a 12:12 h light/dark period was quantified. The white bars in top of the graphs represent the light (ZT0–12) and the black bars the dark (ZT12–24) periods. The VIP mRNA quantities were normalised with the β2-microglobulin mRNA quantities present in the same samples, both were determined by real-time RT-PCR. Each bar represents the mean VIP quantity ± S.E.M. of 7–9 SCNs, respectively. The curves are cosinor-rhytmometry fits to the data points (A: $P<0.01$; B: $P=0.06$).

ZT 5.4) and nadir 12 h later in the middle of the night (ZT 17.2 and ZT 17.4, for WT and PAC_1–/–, respectively). The amplitudes of the oscillations were also similar in WT and PAC_1–/– mice. So during fixed 12h:12h LD cycle, expression of the VIP mRNA does not seem to be dependent on PACAP signalling through PAC_1–/–. During DD, significant rhythmic patterns of the VIP mRNA expression with periods of 24 h in both WT ($P<0.0001$) and PAC_1–/– mice ($P<0.05$) (Fig. 2) were also seen. We have previously shown that PAC_1–/– mice have a significantly shorter free-running period of locomotor activity (τ) in DD compared to WT, $\tau=23.3$ and $\tau=23.7$, respectively (Hannibal et al., 2001). Calculations using their respective τ's instead of 24 h did not change significance levels and resulted in only very minor changes in the times of maximal/minimal expression, amplitude and mean (Table 1).

The phases of the rhythms were delayed in both WT and PAC1–/– animals as compared to the LD cycles. However, the phase-delay was more pronounced in the WT animals, amounting to 9 h as compared to 5 h in the PAC_1–/– mice. Thus, the calculated peak expression of VIP mRNA during DD was observed at ZT 14.1 and at ZT 10.6 in WT and PAC_1–/– mice, respectively. The level of VIP mRNA as well as the amplitude of VIP oscillations was higher in DD than during LD in both the WT and PAC_1–/– animals. The increase in amplitude was more pronounced in SCN of WT mice, being almost four-fold, as compared to the two-fold increase in PAC_1–/– mice.

The present study demonstrates that while identical temporal VIP mRNA profiles were observed in WT and PAC_1 receptor deficient mice during a 12 h/12 h LD cycle two important differences were disclosed during DD. Firstly, the phase-delay of the VIP mRNA rhythm in PAC_1–/– mice was

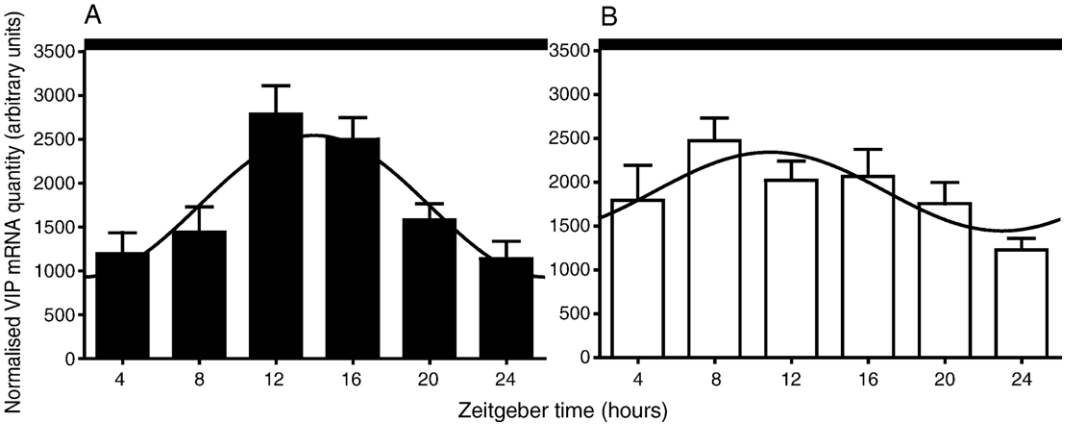

Fig. 2 – Circadian expression of VIP mRNA in SCN from wild type (A) and PAC_1–/– (B) mice. VIP mRNA concentration in SCN of mice sacrificed every 4th hour during a 24 h period in constant darkness (represented by the black bars in top of the graphs) was quantified. The VIP mRNA quantities were normalised with the β2-microglobulin mRNA quantities present in the same samples, both were determined by real-time RT-PCR. Each bar represents the mean VIP quantity ± S.E.M. of 7–8 SCNs, respectively. The curves are cosinor-rhytmometry fits to the data points (A: $P<0.0001$; B: $P<0.05$).

Table 1 – Details of the cosinor analysis of the VIP expression in SCN during LD and DD

	LD		DD			
	τ=24		τ=24		τ=23.7	τ=23.3
	WT	PAC_1–/–	WT	PAC_1–/–	WT	PAC_1–/–
T_{max}	5.2	5.4	14.1	10.5	14.1	10.6
T_{min}	17.2	17.4	2.1	22.5	2.1	22.4
Amplitude	429	455	1616	897	1607	882
Mean	913	1052	1737	1894	1748	1903
Significance	$P<0.01$	$P=0.06$	$P<0.0001$	$P<0.05$	$P<0.0001$	$P<0.05$
R^2	0.20	0.12	0.43	0.15	0.43	0.15

For the data obtained during DD, the calculations have been done both using a phase of 24 h and the actual free running period (τ) of the two genotypes.

much smaller (5 h) than in WT mice (9 h). Secondly, the amplitude of VIP mRNA oscillations was two-fold lower in PAC_1–/– mice as compared to WT mice.

The observation that VIP mRNA in the SCN of both WT and PAC_1–/– mice is rhythmic during DD suggests that it is under control of the circadian pacemaker. Our findings in WT mice during DD accord with a previous study in mice (Dardente et al., 2004). In LD, however, Dardente et al., found the peak of maximal VIP mRNA expression to be later. This discrepancy could be explained by differences in mouse strain or by methodological differences i.e. in situ hybridisation on part of the SCNs in the Dardente study and RT-PCR on entire SCNs in the present study.

The smaller phase-delay of VIP mRNA rhythm in PAC_1–/– mice as compared to WT mice when transferred to DD suggests importance of PAC_1 receptor signalling during this condition. As mentioned, PAC_1–/– mice have a significantly shorter τ in DD as compared to WT (Hannibal et al., 2001), and one might speculate that the less pronounced phase delay of the VIP rhythm and the shorter τ in PAC_1–/– compared to WT mice are related. In addition, PAC_1–/– mice have an impaired ability to phase-delay after light stimulation at early night (Hannibal, 2006). How the changes in the phase of the VIP rhythm in the SCN relate to behaviour remain to be clarified, but the delay in activity rhythm is far smaller than the surprisingly large 9 h delay in VIP mRNA rhythm appearing already during the second cycle in DD. Nevertheless, the phase delay was significantly blunted in the PAC_1–/– mice suggesting that PACAP signalling—most likely via the RHT is involved. However, as the delay in VIP rhythm was not completely abolished in PAC_1–/– mice, PACAP signalling via $VPAC_2$ receptors in the SCN (Reed et al., 2002) and/or other transmitters must be involved as well.

During DD, the VIP expression increased in SCN of both WT and PAC_1–/– animals. Light has previously been shown to exert a negative effect on the VIP expression in the SCN of rats (Albers et al., 1987; Shinohara et al., 1998, 1999). A similar effect of light on the VIP expression could be the reason for the lower levels observed during LD cycles as compared to DD. Both, the level of VIP mRNA as well as the amplitude of the oscillation was higher in DD than during LD. The increased amplitude was however much more pronounced in the SCN of WT mice, being almost four-fold compared to the two-fold increase in PAC_1–/– mice supporting that PAC_1 signalling plays a role in the VIP gene expression in the SCN. In conclusion, PACAP signalling to the SCN via PAC_1 seems to be important for the delay in the phase of the VIP mRNA rhythm observed in DD as compared to LD cycles.

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