Initial demonstration of rhythmic Per gene expression in the hypothalamus of a non-mammalian vertebrate, the house sparrow

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Received 31 December 2000; accepted 3 February 2001

In mammals, the major pacemaker controlling circadian rhythmicity is located in the hypothalamic suprachiasmatic nuclei which are characterized by specific molecular features including the expression of three homologues of the Drosophila clock gene period (per). Until now, no comparable structure has been unambiguously described in the brain of any non-mammalian vertebrate. We cloned the PAS-domain of the Per2 gene in the house sparrow (Passer domesticus), a model organism in circadian research. Hypothalamic expression of passerPer2 (pPer2) showed a marked diurnal rhythm in the suprachiasmatic nucleus, a cell group located in the anterior hypothalamus directly above the optic chiasm and adjacent to the third ventricle. Additionally, pPer2 was diurnally expressed in the lateral hypothalamus. This first demonstration of rhythmic clock gene expression in the hypothalamus of a non-mammalian vertebrate provides basic information for future research on the evolution of circadian pacemaking systems.

Key words: Birds; Lateral hypothalamus; Passer domesticus; pPer2; Rhythm; SCN: Suprachiasmatic nucleus

INTRODUCTION

Circadian rhythms, i.e. daily rhythms of endogenous origin persisting for many cycles even under constant environmental conditions, are a fundamental basis for the behaviour and physiology of all higher organisms [1,2]. These rhythms are generated within cells containing a specific ‘molecular clock’ consisting of autoregulatory transcriptional and translational feedback loops [3]. Aggregations of such clock cells may form functional units acting as interfaces between organism and environment to synchronize animals to external timing cues and to drive rhythms in biochemical, physiological and behavioural functions. In mammals, the master clock controlling circadian rhythmicity is located in the hypothalamic suprachiasmatic nuclei (SCN) which are characterized by distinct anatomical, physiological and neurochemical features [4,5] as well as the presence of specific transcription factors and clock genes, particularly three homologues (Per1, Per2, Per3) of the Drosophila clock gene period (per) [6].

Until now, no comparable structure has been unambiguously described in the brain of any non-mammalian vertebrate. Similar to mammals, the SCN of birds is located in the anterior hypothalamus adjacent to the third ventricle [7–9]. About 20 years ago, experimental lesioning studies in birds suggested that this SCN might represent the functionally equivalent structure to the mammalian SCN since lesions disrupted circadian behaviour [10–12]. Since then, several hypothalamic cell groups have been assumed to represent the central nervous circadian pacemaker in birds and the term SCN has been applied to more than one nucleus (reviewed in Norgren and Silver [13]). Anatomical and lesioning studies in birds suggested the circadian oscillator to be located in the SCN while others argued that the avian circadian oscillator might be located in a small retino-recipient cell group in the lateral hypothalamus, the so-called lateral hypothalamic retinorecipient nucleus or visual SCN [7–14]. However, neither neurochemical nor functional studies allowed final conclusions and whether birds possess a single nucleus homologous to the mammalian SCN remained controversial [13,14]. Several reasons may have contributed to the lack of detailed knowledge about the avian equivalent of the mammalian hypothalamic circadian oscillator, including incomplete and inconsistent anatomical descriptions of the avian hypothalamus, confusion in delineation as well as terminology of cell groups, and the lack of a molecular approach defining key genes and/or transcription factors of circadian oscillators. Recently, two Per genes (qPer2, qPer3) have been cloned in the Japanese quail and demonstrated to be rhythmically expressed in two structures containing circadian oscillators, the retina and the pineal gland [15]. To investigate whether characteristic components of a molecular circadian clock...
are detectable in the avian hypothalamus we have chosen the house sparrow (Passer domesticus), a model organism in circadian research [11,16,17].

MATERIALS AND METHODS
Adult house sparrows were kept in individual activity-recording cages for ≥2 weeks prior to experimentation under a 12:12 light:dark schedule. One bird was killed at Zeitgeber time (ZT) 6 (mid of day) by decapitation and its brain quickly removed for RNA isolation (RNaZol B protocol from WAK-Chemie Medical GmbH, Germany). RT-PCR was performed with primers specific for the PAS-domain of the quail Per2 gene [15] (Accession number AB029890): forward primer 5'-AATGCAGATATGTTTG CTGTGC-3', reverse primer 5'-TGAAACTGGACCAGCT AGTGTC-3'. The resulting PCR product of 688 nucleotides in length was cloned into the pcRII-TOPO vector using the TOPO TA cloning system (Invitrogen, USA). The partial P. domesticus Per2 (pPer2) cDNA was sequenced and deposited in GenBank (Accession number AY 007259).

For in situ hybridization, house sparrows were given an overdose of anesthetic and transcardially perfused with fixative (4% paraformaldehyde in phosphate buffered saline; Sigma, USA). To investigate the anatomical localization and a possible rhythm of the expression of pPer2, in situ hybridization was carried out with house sparrow brains (n=8) as described previously for mouse [18] at four different times (ZT 6, 12, 18, 24) during a 12:12 light-dark cycle. To avoid further confusion in terminology, naming of hypothalamic cell groups follows Kuenzel and van Tienhoven [9].

RESULTS
The amino acid sequence of the PAS domain in the house sparrows Per2 gene is 96% identical to Per2 of the Japanese quail (qPer2) [15] and shows 66%, 77%, and 56% identity to mouse Per1, Per2 and Per3, respectively [19–22]. Similar to what has been reported for Per2 in Japanese quail [15], expression of pPer2 was found in the retina and in the pineal gland of the house sparrow. Additionally, pPer2 expression was detected in the preoptic nucleus, in the suprachiasmatic nucleus (SCN), as well as in the lateral hypothalamus (Fig. 1). No remarkable pPer2 expression was found in any other hypothalamic region or any other part of the brain investigated. No diurnal changes in pPer2 expression could be observed in the preoptic nucleus although slight variations between the time points were detectable. A marked diurnal rhythm of pPer2 was found in the suprachiasmatic nucleus as well as in the lateral hypothalamus. At ZT 24 (shortly before lights on), a strong pPer2 signal was visible exclusively in the most rostral portion of the SCN (Fig. 1a). At ZT 6 (midday), pPer2 was strongly expressed throughout the longitudinal extension of the SCN (Fig. 1a,b,c) and in the lateral hypothalamus (Fig. 1c). AT ZT 12 (shortly before lights off), pPer2 levels were low in the SCN as well as in the lateral hypothalamus (Fig. 1b,c). pPer2 expression was indistinguishable from background levels in the SCN as well as in the lateral hypothalamus at ZT 18 (midnight; Fig. 1a,b,c).

DISCUSSION
Our data provide initial evidence for the expression of a period homolog in the hypothalamus of a non-mammalian vertebrate, the house sparrow. Hypothalamic expression of pPer2 was strongest and showed a marked diurnal rhythm in the SCN, a cell group characterized by a distinct aggregation of neurons located in the anterior hypothalamus directly above the optic chiasm and adjacent to the third ventricle [7–9]. Additionally, pPer2 was expressed in a group of cells in the lateral hypothalamus anatomically corresponding to the so-called lateral hypothalamic retinocryptic nucleus (LHRCN) [13] or visual SCN [14], as well as dorsal to the LHRCN in a group of cells located in the lateral hypothalamic area. The temporal expression pattern of the pPer2 gene was not homogenous in these distinct locations but high expression levels were found between ZT24 and ZT6 in the rostral SCN (similar to what has been described for mPer1 in the SCN of the mouse [3]) and between ZT6 and ZT12 in the medio-caudal SCN as well as in the lateral hypothalamus (similar to that shown for mPer2 in the SCN of the mouse [3]).

Several functional attempts have been made to characterize the avian equivalent to the mammalian hypothalamic circadian oscillator during the last 20 years. For example, lesions of the SCN resulted in disruptions of circadian activity [10–12], but lesions of the lateral hypothalamus did not affect activity rhythms [23]. Additionally, the immediate early gene c-fos, expressed in response to light in the mammalian SCN exclusively at phases of the circadian rhythm during which light can shift the rhythm [24], was found to respond to light in the lateral hypothalamus but not in the SCN of birds and expression did not vary depending on the time of day or in relation to the effect of light on phase shifts [25]. Our data in the house sparrow provide further insight by showing that rhythmic Per2 expression in the hypothalamus is not restricted to a single nucleus, as it is the case in mammals [3,6,26], but can be found in both structures that have been assumed to represent the avian hypothalamic circadian oscillator, the SCN and the lateral hypothalamus. Additionally, the temporal regulation of pPer2 appears to differ since it was expressed during late night (ZT24) and at midday (ZT6) in the rostral SCN but during the second half of the day (ZT6 and ZT12) in the medio-caudal part of the SCN, as well as in the lateral hypothalamus. The occurrence of a strong pPer2 signal in the most rostral portion of the SCN before lights on suggests an endogenous circadian oscillator to be involved in the regulation of pPer2 expression. The rhythm of pPer2 in the lateral hypothalamus could be driven by light, by a circadian oscillator, or by light as well as a circadian oscillator since pPer2 was only visible during the light phase.

Mouse Per genes, particularly mPer2, have been shown to be substantial components of the hypothalamic circadian clock in mammals [26]. If pPer2 had similar functions to mPer2, our findings might suggest either the presence of subpopulations of clock cells in the SCN and the lateral hypothalamus with different temporal organizations or a spatio-temporal expansion of pPer2 from the rostral SCN to the medio-caudal SCN as well as to the lateral hypothalamus under the influence of a circadian oscillator and/or light. Hence, apart from light, pPer2 expression in the
hypothalamus could be regulated by an intrinsic hypothalamic circadian oscillator or even by the pineal gland, a well-characterized circadian oscillator and pacemaker in the house sparrow [17,27].

CONCLUSION

Our data are the first demonstration of rhythmic Per gene expression in the hypothalamus of a non-mammalian vertebrate and support the idea of a complex organization of the avian hypothalamic circadian oscillator. This is in accordance with the high complexity of the avian circadian pacemaking system as a whole [28]. Whether the rhythm in pPer2 expression indeed reflects the activity of one or several hypothalamic circadian oscillators remains to be established. However, our data provide first molecular insight into an issue that is under debate since decades and provide basic information for future research on the evolution of vertebrate circadian pacemaking systems.

REFERENCES

Acknowledgements: Financial support by the German Science Foundation (grants no. Br-1899/1-1 and AL549/1-1) is gratefully acknowledged. Our thanks are due to T. Yoshimura and S. Ebihara for providing information about qPer2 prior to publication.