Supplementary Materials

"Identification of neuromedin S and its possible role in the mammalian circadian oscillator system" by K. Mori *et al*.

Supplementary Data

Bioinformatic analysis of the neuromedin S (NMS) gene

To analyze the structure of the *NMS* gene, public genome databases were searched using the full-length sequence of mouse NMS cDNA as a query sequence. The NMS genomic sequence was identified in one genomic contig of mouse chromosome 1 (RefSeq accession number NT_080070). The mouse *NMS* gene was ~11 kbp in length, which is ~11 times the length of the cDNA. The gene was composed of 10 exons interrupted by 9 introns (see Supplementary Figure S1A). The nucleotide sequences at the 5'-donor and 3'-acceptor sites of all introns conformed to the GT/AG rules (Shapiro & Senapathy, 1987) (see Supplementary Table SI). The structures of the rat and human *NMS* genes were also analyzed, and all exon-intron boundaries were conserved among mouse, rat and human (see Supplementary Tables SII and SIII).

The transcription initiation site of the mouse *NMS* gene was identified by RNA ligase-mediated rapid amplification of cDNA ends (RACE) PCR, which is able to determine the 5'-end of full-length mRNA. 5'-RACE PCR yielded a single product. The sequencing of this product showed that the initiation site is 137 bp upstream of the ATG translation initiation codon (see Supplementary Figure S1B). The first 400 bp of the 5'-flanking region of the *NMS* gene shows particularly high sequence identity (> 71%) among mouse, rat and human (see Supplementary Figure S1B), and regions further upstream are also conserved in some places. Computational analysis showed that there is no TATA-box or CCAAT sequence adjacent to the transcription initiation site. These genes share several putative binding sites for transcription factors including GATA-1 (Merika & Orkin, 1993), Oct-1 (Groenen *et al*, 1992), Sox-5 (Denny *et al*, 1992) and

AP-1 (Rauscher *et al*, 1988) in the highly conserved 5'-flanking region (see Supplementary Figure S1B). Neither a cAMP-responsive element nor a CACGTG E-box sequence was found within the first 3 kbp of the 5'-flanking regions of the mouse, rat and human *NMS* genes. These two putative binding sites are involved in photic-induction and intrinsic circadian rhythmicity of gene expression, respectively (Reppert & Weaver, 2001).

Supplementary Materials and methods

Computational analysis of the NMS gene

The sequences of the *NMS* genes were obtained from genome databases using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST). Exon sequences and exon-intron boundaries were defined by comparison with cDNAs and by the GT/AG rule. The putative binding sites for transcription factors were analyzed using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) with the TRANSFAC database (Heinemeyer *et al*, 1998).

Chick rectum contraction assay

The chick rectum contraction assay was performed according to the method of Currie *et al*, (1983). Contractile activity was measured using strips of freshly isolated chick rectum in 3 ml of Krebs-Henseleit solution at 37 ˚C.

Measurement of blood pressure

Systemic blood pressure was continuously monitored in Sprague-Dawley rats anesthetized with pentobarbital (50 mg/kg) from the carotid artery as described previously (Minamino *et al*, 1985). Test samples were dissolved in 100 μl of saline and administered through a catheter in the jugular vein.

Intranuclear administration of NMS into the suprachiasmatic nucleus (SCN)

Intranuclear administration into the SCN was performed according to the method of Piggins *et al*, (1995) with slight modifications. Rats were allowed to run free under constant darkness for 2 weeks. Each rat was then implanted with a 26-gauge stainless steel guide cannula projecting to the SCN according to the coordinates of Pixinos & Watson (1998). Test samples were dissolved in 1 μl of saline and administered via a 31-gauge stainless steel injector inserted to extend 1 mm below the end of the guide cannula. Locomotor activity was recorded for 2 weeks before and after administration as described previously (Nakahara *et al*, 2004). Correct cannula placement was confirmed by histological analysis at the end of the experiment period. Rats received an injection of 1 μl India ink to mark the injection site, and were then decapitated. The brains were immediately frozen, sliced on a cryostat into 20 μm coronal sections and stained with crystal violet. Sections were compared to the corresponding sections from the rat brain atlas (Paxinos & Watson, 1998). Data were excluded when the injection site extended more than 200 μm outside the SCN.

Supplementary References

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