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Short communication

Circadian variation of nitric oxide synthase activity and cytosolic protein levels in rat brain

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Abstract

The circadian variation of nitric oxide synthase (NOS) activity and cytosolic protein content in the cerebellum, brainstem, hypothalamus, hippocampus, and the remainder of the brain were studied in rats. Both NOS activity and cytosolic protein concentrations were the highest during the dark period and lowest in the light period. Hypothalamic NOS activity exhibited the most pronounced change in activity with time increasing by ~ 120% from mid-light to mid-dark.

Keywords: Nitric oxide synthase; Nitric oxide; Circadian rhythm; Sleep; Central nervous system; Hypothalamus; Protein level; Rat

Nitric oxide (NO), a free radical produced by the oxidation of L-arginine by nitric oxide synthase (NOS), is a key messenger in many physiological responses. NO produced in the endothelia induces smooth muscle relaxation, NO produced by macrophages participates in immune responses, and NO in the central nervous system (CNS) is involved in neural functions such as synaptic plasticity and memory formation. NO is also implicated in sleep regulation and regulation of circadian functions [reviewed in [22]]. Inhibition of NO production by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) suppresses non-rapid-eye-movement sleep (NREMS) in rats and rabbits and interferes with the somnogenic effects of interleukin-1 (IL-1) in rabbits [16]. Conversely, administration of NO donors *S*-nitroso-*N*-acetylpenicillamine and molsidomine (SIN-1) elicits increases in NREMS in rats [15]. NO is also posited to mediate circadian phase shifts in the suprachiasmatic nucleus in response to NMDA receptor activation [8,25]. To further elucidate the role of NO in CNS regulation, we have investigated the circadian cycling of NOS activity and cytosolic protein content in five different brain regions in rats. The results indicate that brain NOS activity and total cytosolic protein levels have a clear circadian rhythm with highest levels found during the dark period. A part of the present results was previously published in abstract form [2].

Twenty-eight male Sprague–Dawley rats were habituated for two months to a 12:12 h dark/light cycle (lights on at 09.00 h). Four animals were kept per cage and the rats remained with the same cage-mates for the entire habituation period. Food and water were provided ad libitum. Ambient temperature was 21 ± 1°C. On the experimental day, 6 animals, one from each cage, were decapitated at the following time points: 15.00 h, 21.00 h, 03.00 h and 09.00 h. Brains were immediately removed and dissected into five regions: cerebellum, brainstem, hypothalamus, hippocampus, and the remainder of the brain after dissections ('rest of brain'). Brain regions were immediately weighed and washed in ice-cold homogenizing buffer containing 0.32 M sucrose, 20 mM HEPES, (pH 7.2), 0.5 mM EDTA and 1 mM dithiothreitol. All reagents used in these experiments were purchased from Sigma unless noted otherwise. The samples were kept at 4°C throughout the entire tissue preparation. Brain samples were sonicated in homogenizing buffer and centrifuged (20,000 × g/20 min/4°C). Supernatants were removed and used for assaying NOS activity and obtaining total cytosolic protein concentrations.

In a separate set of experiments we compared cytosolic protein concentrations from brain samples of perfused and non-perfused rats. At 03.00 h, two rats were anesthetized with 87 mg/kg ketamine and 13 mg/kg xylazine and

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perfused with 120 ml of 0.9% NaCl prior to decapitation. Another two rats were decapitated without perfusion. Brain dissection and tissue preparation were as described above.

NOS activity was measured by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline [6]. Briefly, endogenous arginine was removed by applying 2 ml of each supernatant to a 1 ml Dowex 50WX-8 (H^+ form) column. The pH of the column was titrated to 7.2 with 10 M NaOH and the columns were pre-equilibrated in homogenizing buffer. Total assay volume was 400 ml and each sample was run in triplicate. 340 μl of enzyme extract was incubated for 45 min at 37°C in buffer containing 1 mM NADPH, 1 mM dithiothreitol, 50 mM HEPES (pH 7.2), 1.25 mM $CaCl_2$, 100 nM [^{3}H]arginine (35 Ci/mmol) final concentrations. Reactions were stopped by the addition of 2 ml of stop buffer (20 mM HEPES, pH 5.5 and 2 mM EDTA). Each reaction mixture was applied to a 1 ml Dowex 50WX-8 column (pH titrated to 5.5 with 10 M NaOH and pre-equilibrated with stop buffer) and then eluted with 2 ml of water. Aliquots of the elutant (150 μl) were quantified for [^{3}H]citrulline by liquid scintillation. NOS activity is expressed as [(sample count × 100)/(total count)]/g wet tissue or mg protein. Total cytosolic protein concentrations were measured from 10 μl fractions of supernatant in triplicate by standard bicinchoninic acid assay method (Pierce, Rockford, IL) on microtiter plates.

Comparisons were made between the NOS activities or protein contents of the five brain regions across the four time points by using two-way ANOVA. When ANOVA indicated significant effects, Student's *t*-test was used a posteriori to compare NOS activities/protein levels between two time points or two different brain regions. An alpha-level of $P \leq 0.05$ was taken as indicating statistical significance.

There were significant differences in NOS activity among brain regions, and in all five regions NOS activity exhibited significant changes with time (Fig. 1A; ANOVA on values of NOS activity/g wet tissue; differences among brain regions: $F_{4,98} = 122.1$, $P < 0.001$; time effect: $F_{3,98} = 16.4$, $P < 0.0001$).

The cerebellum showed the highest NOS activity of all brain regions assayed, 79.9 ± 6.32 to 108.4 ± 5.91 unit NOS activity/g wet tissue, hypothalamic activity was the second highest, hippocampal and 'rest of the brain' activities were approximately the same, and the brainstem contained the lowest activity levels, 16.2 ± 2.12 to 28.4 ± 2.62 unit/g. (Post hoc *t*-test: significant differences between any two brain regions, except between 'rest of the brain' and hippocampus, and between hippocampus and brainstem; significant differences between any two time points, except between 03.00 h and 09.00 h.)

Within each brain region there were significant changes of NOS activity with time. NOS activity in all brain regions assayed was higher during the dark phase and lower in the light period. The most prominent change occurred in the hypothalamus. Hypothalamic activity increased by ~120% between 15.00 h and 03.00 h. All brain regions, except the cerebellum, had the highest NOS activity at 03.00 h, the midpoint of the dark phase, and NOS activity in the cerebellum also increased during the dark in comparison to the light period. The circadian changes in NOS activity cannot be attributed simply to a general increase in protein synthesis because when NOS activity is normalized to protein content the circadian changes in NOS activity remain significant (Fig. 1B; differences between brain regions: $F_{4,96} = 68.4$, $P < 0.0001$; time effect: $F_{3,96} = 5.5$, $P = 0.0016$).

There was a significant difference in the cytoplasmic protein content among the five brain regions and significant changes in cytosolic protein concentration during the 24-h day (Fig. 2; ANOVA across four time points and five brain regions; brain region effect: $F_{4,96} = 36.01$, $P < 0.0001$; time effect: $F_{3,96} = 17.69$, $P < 0.0001$). In general, all areas, with the exception of the 'rest of the brain'

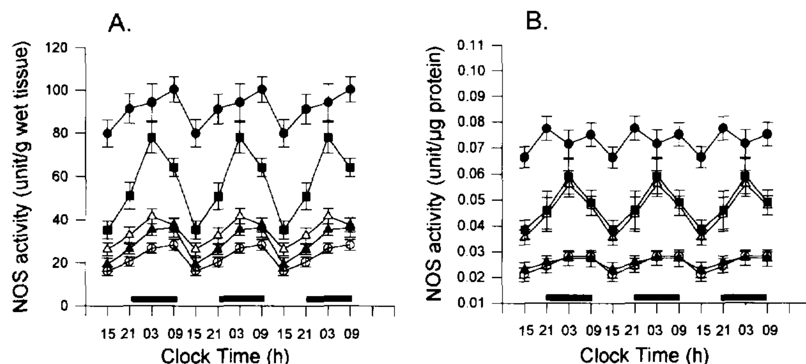

Fig. 1. Circadian changes in NOS activity. Data from one 24-h experimental period are triple-plotted. Each time point represents the mean of 5–6 samples. Error bars indicate S.E.M. Dark horizontal bars indicate the dark period of the day. Hippocampus (solid triangle); 'rest of the brain' (open triangle); cerebellum (solid circle); brainstem (open circle); hypothalamus (solid square). A: NOS activity is expressed as [(sample count × 100)/(total count)]/g wet tissue. NOS activity showed significant changes with time in all brain regions with an overall significant difference among NOS activities of different regions. In general, NOS activity increased during the dark phase and decreased during the light phase. B: NOS activity is expressed as [(sample count × 100)/(total count)]/mg protein. Circadian variation of NOS activity remains significant when normalized to protein content.

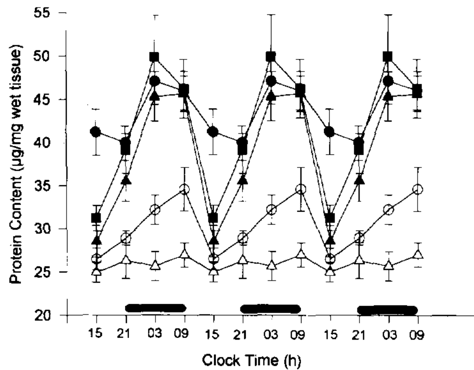

Fig. 2. Circadian variation of brain cytosolic protein content. See legend to Fig. 1 for details. There is an overall significant difference in protein content in all brain regions, and a significant increase in protein content in all regions, except 'rest of the brain', during the dark period.

showed significant increases in cytosolic protein content during the dark period. At 15.00 h the cerebellum contained the highest amount of protein, 41.2 ± 2.65 mg/mg wet tissue, and at 03.00 h the hypothalamus contained the highest amount, 49.8 ± 4.78 mg/mg wet tissue. The lowest protein content was measured in the 'rest of the brain' which did not show circadian variation. Brainstem cytosolic protein levels, while significantly higher than 'rest of the brain', were significantly lower than the other three regions during the dark period. Hypothalamic and hippocampal protein levels demonstrated the largest circadian variation increasing from 31.2 ± 1.47 mg/mg at 15.00 h to 49.8 ± 4.78 mg/mg at 03.00 h and 28.5 ± 1.83 mg/mg at 15.00 h to 45.3 ± 2.83 at 03.00 h, respectively.

The concentration of plasma proteins are higher during the dark period compared to the light period in rats [4]. To determine whether increased plasma protein levels contribute to the increase in brain cytosolic protein concentrations during the dark period, cytosolic protein levels from perfused and non-perfused brains were compared at 03.00 h. The cytosolic protein levels in the perfused brain did not differ from those measured in the non-perfused animals (data not shown). This indicates that in our assay system plasma proteins do not significantly contribute to the measured protein levels of the brain during the dark period.

Our results confirm previous findings that the cerebellum contains the highest amounts of NOS catalytic activity and the brainstem contains low levels of NOS activity in rat brain [5,7]. We have also demonstrated that NOS activity in the rat brain peaks in the middle of the behaviorally active period and reaches lowest activity levels in the middle of the rest period. Similarly, levels of cGMP, a second messenger produced by NO activation of guanylyl cyclase, is the highest during wakefulness in the hippocampus, mid-brain, pons-medulla and cerebellum [18]. While NOS activity changed in all five regions measured, the most pronounced change was observed in the hypothalamus. This may be related to the possible role of NO in the regulation of circadian rhythms in the suprachiasmatic nucleus [8,25]. Alternatively, increased NOS activity during the behaviorally active period may reflect a general increased neural activity since NO production has been shown to be coupled to neural activation [3,9–11] and during wakefulness there is a global increase in neural activity in comparison to NREMS [12].

There are three primary isoforms of NOS. Two constitutively expressed Ca^{2+}/calmodulin-dependent isoforms are found primarily in the endothelia and neurons. An inducible, Ca^{2+}-independent form is found in macrophages and glia. All three isoforms can be expressed in the brain (reviewed in [22]). It is not known which isoform(s) account for the elevation of NOS activities during the night. The endothelial isoform is predominantly membrane bound. In our assay we measured NOS activity from the cytosolic fraction; therefore, the contribution of endothelial NOS in the observed changes in brain NOS activity is likely to be relatively minor.

The finding that NOS activity is high during the behaviorally active circadian phase is consistent with the hypothesis that NO is involved in sleep regulation. Previously we showed that the injection of the NO donor, SIN-1, at the beginning of night induces NREMS after a latency of ~ 9 h [15]; this suggests that there is a relatively slow biochemical cascade behind the somnogenic effects of NO. NO induces the expression of immediate early genes, c-*fos* and *junB*, which regulate transcription [13,19]. Thus, it is possible that the accumulation of gene products stimulated by NO is necessary for mediating the somnogenic effects of NO. High NOS activity during the night is consistent with the idea of delayed cascade-type events being involved in NO-mediated sleep. However, if NOS activity is blocked by L-NAME in rats, sleep is suppressed within one hour [14]; the reasons for these apparent discrepancies is unknown, though it is reasonable to propose both immediate effects of NO on neurons and more long-term sleep effects involving gene transcription are involved in sleep regulation.

Although NOS activity was high in the behaviorally active period, it is possible that NO itself may be elevated during the rest period in some brain structures. The time course of changes in NOS activity does not necessarily reflect the exact time course of NO activity itself since NO may be carried and stored by proteins. NO^+ binds rapidly to thiol groups to cause the *S*-nitrosylation of proteins, and it can be released gradually [24]. In fact, in plasma NO is carried and stored by albumin [23]. Furthermore, in the present experiments, NOS activity was measured from relatively large brain regions, and in more circumscribed areas, circadian changes in NOS activities may exhibit a different time course. Thus, any small variation of NOS activity associated with a specific sleep response in those regions may be masked by large background levels of NOS activity.

Our results also demonstrated a circadian variation in cytosolic protein levels in the cerebellum, brainstem, hypo-

thalamus, and hippocampus, with highest levels reached in all regions during the dark period. Similarly, plasma protein levels and protein content of the supraoptic neurons are increased during the dark in rats [4,20]. Protein synthesis is increased in the suprachiasmatic nuclei and paraventricular nuclei during the light period in rats [1] and in the cerebellum during NREMS [21]. Increased protein synthesis however, does not necessarily correlate with net protein levels since protein content is a function of both synthesis and degradation. Because cytosolic fractions contain primarily regulatory proteins and fewer structural peptides, higher levels of cytosolic proteins during wakefulness may be due to increased metabolic activity of the neurons. The activities of enzymes involved in metabolism increase in activity in the brain during the dark period [17] and c-*fos* mRNA expression, a non-specific indicator of neural activity also increases in the brain during the activity period of the circadian cycle [12].

In conclusion, the finding of a circadian variation in basal levels of NOS activity is consistent with the hypothesis that NO-receptive mechanisms are involved in sleep regulation.

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