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EFFECT OF THE PHOTOPERIOD ON THE GLUTAMATE LEVEL IN THE SUPRACHIASMATIC NUCLEUS OF PREGNANT AND NON-PREGNANT RABBITS

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Abstract: Microdialysis sampling was used to measure the glutamate level (GLU) contained in the suprachiasmatic nucleus (SCN) in 10 pregnant and 10 non-pregnant, subjected to either a 16:8 h light-dark cycle or a 12 h advanced photoperiod. Results revealed that GLU levels were significantly greater in samples taken for 2 h (15 min intervals), −60, −45 and −30 min before parturition ($P<0.009$, $P<0.04$, $P<0.02$), and +45 and +60 min after birth ($P<0.009$, $P<0.05$) respectively, in pregnant rabbits subjects to a 16:8 LD photoperiod (with light from 6:00-22:00 h). Parturition in these animals occurred at day 31 of gestation. Pregnant rabbits exposed to 12 h advanced photoperiod showed lower GLU levels in the SCN, and a longer gestation period and parturition process. In non-pregnant rabbits the GLU levels in the SCN were also significantly higher ($P<0.009$, in all samples) in comparison with rabbits subjected to the 12 h shifted photoperiod. It is possible that the photoperiod may have influenced GLU levels, and consequently, the length of gestation, the number of young and the time of birth. However, further studies involving new biotechnologies and a greater number of animals are needed to confirm these results.

Key words: photoperiod, pregnancy, suprachiasmatic nucleus, glutamate, rabbits.

INTRODUCTION

It is now well documented that the pacemakers, the pathways to overt rhythms and the mechanisms of entrainment are all part of the circadian system underlying not only the internal temporal organization of multiple functions, but also the remarkable capacity of organisms to measure the passage of time in a precise and flexible manner. Robert Moore (1983) reported that the destruction of the suprachiasmatic nucleus (SCN) of the hypothalamus, not only prevents the entrainment of circadian rhythms to light, but also disrupts various endogenous behavioral and hormonal circadian rhythms (Bos, 1990). Therefore, the SCN may house self-contained circadian oscillators, and the retinohypothalamic pathway (RHT) may serve to couple the mammalian circadian system to the external light-dark cycle (Moore, 1983; Jilge, 1993). Light stimulation of the retina results in the direct secretion of the excitatory neurotransmitter glutamate (GLU) from the RHT pathway in the SCN (Abrahamson and Moore, 2001; Ebling, 1996; Mikkelsen *et al.*, 1992). Although no information is available on the neural control of light entrainment in rabbits, this presumably also involves the RHT and the SCN, as is now well established with regards to a variety of rodent and primate species (Lincoln and Porter, 1976; Meijer, 2001; Reppert and Weaver, 2002). Wild rabbits are essentially nocturnal and display a clear daily pattern of circadian activity and show a marked photoperiodic organization of parturition and nursing (Jilge, 1993). They usually give birth during daylight hours with parturition generally lasting no more than 10 min for the birth of up to

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almost 14 young rabbits (Fuchs and Dawood, 1980; Hudson *et al*., 1999). Needless to say, the developing embryos and fetuses are subjected to a hormonal intrauterine environment; however the process triggering parturition is still not completely understood.

In order to investigate a possible relation between the photoperiod and time of birth in rabbits, our laboratory work was twofold: firstly we examined the diurnal pattern of peripheral events during late pregnancy and uterine activity across the 24 h day, i.e., during the light-dark phase, while the second project studied the level of the classic neurotransmitter - glutamate - in the SCN, the hypothalamic nucleus involved in both circadian and reproductive processes. In a previous report, our laboratory found that the uterus of the pregnant rabbit presents a diurnal rhythm and modifies its sensitivity to oxytocin during the daylight hours, when parturition normally takes place (Ninomiya *et al.*, 2004).

Therefore, in this set of experiments, our objective was to measure the GLU level in the SCN of pregnant and non-pregnant rabbits during two light photoperiods: 16:8 h light-dark cycle and a 12 h shifted photoperiod.

MATERIALS AND METHODS

The experimental procedures were conducted according to the Guiding Principles for Research Involving Animals and Humans at the School of Medicine, UNAM.

Animals and surgery

Twenty adult New Zealand white rabbits, 10 pregnant and 10 non-pregnant, weighing 3.5-4.5 kg were used for this study. They were bred and kept in the animal housing facilities of the School of Medicine, on a 16:8 light-dark cycle at 20-22º C with food (Purina rabbit chow) and water *ad libitum*. The animals were fed and cleaned between 9:00 and 11:00 h. The light-dark cycle was chosen to correspond approximately to the natural conditions present during the rabbit breeding season. Two weeks before mating, animals were placed in the light-dark cycle appropriate for their particular treatment group (see experimental groups). Mating took place at 12:00-13:00 h and was confirmed by the presence of sperm in vaginal smears taken immediately thereafter.

The implantation of an intracerebral cannula was performed at day 27 of gestation. The pregnant animals were anaesthetized with 10 mg/kg zolazepam i.m. (Zoletil, Virbac, Lab. France). Next, under sterile conditions, they were placed on a stereotaxic frame and implanted with a stainless steel guide cannula, with one internal protective stainless steel gauge, covered with an upper stopper to ensure the cannula remained permeable (Cat. No. 8309010 CMA/10 Microdialysis AB, Stockholm, Sweden). The guide was aimed at the SCN. The guide tubes were cemented in place using dental acrylic and protected with a surrounding plastic ring. Three weeks following the adjustment of the light-dark cycle, the non-pregnant rabbits were implanted with guide tubes aimed at the SCN and five days thereafter were subjected to the microdialysis probe.

Experimental groups

Pregnant animals

Group 1. These animals (n=5) were maintained at the 16:8 h light-dark cycle with lights on from 6:00 to 22:00 h. At full term (day 31 of gestation), animals were prepared for the microdialysis probe.

Group 2. In this group (n=5) the period of light was advanced by 12 h, 3 wk before mating (with lights on from 18:00 to 10:00 h). Animals received the same treatment as those from Group 1 on day 31 of gestation. During the experiment, the eyes of the rabbits were covered with black tape during the dark period in an attempt to reduce any possible influence from the room's artificial lighting.

Estrous animals

Group 1A. These animals (n=5) were maintained at the 16:8 h light-dark cycle with lights on from 6:00 to 22:00 h, for 3 wks prior to preparation for implantation and the microdialysis probe.

Group 2A. For this group (n=5) the photoperiod was advanced by 12 h (light from 18:00 to 10:00 h), 3 wks prior to implantation. And the rabbits were subjected to the same treatment as Group 1A.

Microdialysis sampling experiments

As previously specified, the pregnant animals were anaesthetized at day 31 of gestation. Microdialysis sampling started at 7:00 h and finished 1 h after parturition for all the animals. However for the statistical analysis, only those samples obtained 1 h prior to parturition, during the actual birth and 1 h post-parturition were used. Once the protective gauge was removed microdialysis probes (CM/10 probes, a stainless steel gauge of 20 mm shaft length and 2 mm membrane length) were inserted via the cannula guide into the SCN of each animal. Probes were fixed to the guide tube by means of battery-driven syringe pumps (MS16A, Grasebt Medical, Watford, UK) which delivered Ringer solution (pH 7.4) at a rate of 2.5 µl/min. Samples were collected at 15 min intervals in 500 µl Eppendorf tubes connected to the outflows of the microdialysis probes. In order to prevent oxidation of the recovered substances each Eppendorf tube contained 2 µl of 10% HCl. Depth coordinates were calculated prior to the sampling with reference to the stereotaxic atlas of the New Zealand rabbit brain, (Urban and Richard, 1972) and the rat brain stereotaxic atlas coordinates (Paxinos and Watson, 1998), (Bregma AP=5.5, L=56.4, H=58.6 mm) and adjusted at the time of surgery.

The samples were frozen at −70º C for subsequent analysis through HPLC (Turbochrom 4.1) for GLU, 20 µl were used on a steel LiChrospher 100 RP-18 (5 µm) column (Merck KgaA Germany). Samples were collected until the animals had given birth and for one h post-parturition. The rabbits were then given a lethal dose of the barbiturate – sodium pentobarbital (approximately 60mg/kg as recommended for veterinary use, Pfizer, S.A. de C.V. Mexico) and transcardially perfused with 10% formalin. Following perfusion, the brains were immediately removed, cryoprotected in 10% sucrose, and sectioned coronally at 50 µm (anterior to posterior, at the optic chiasma level) using a vibratome (Reichert USA) and stained neutral red. Anatomical identification of the site of the microdialysis membrane was accomplished with reference to the stereotaxic atlas of the New Zealand rabbit brain and the rat brain with stereotaxic coordinates.

Statistics

For the statistical analysis, mean concentrations of glutamate from the microdialysis samples were calculated for each of the 20 animals, 10 pregnant and 10 non-pregnant estrous rabbits. Samples were taken 1 h prior to parturition, at birth and during the first postpartum h, at 15 min intervals. Samples from non-pregnant animals were taken at the same time intervals. The mean concentration of GLU was then calculated for samples taken in periods with normal or advanced photoperiods. For each glutamate measure, the mean concentration for all the above sampling periods and conditions was subjected to an overall non-parametric test - the Mann Whitney test.

RESULTS

Neuroanatomical localization of microdialysis probes

Figure 1 illustrates the schematic section from a rabbit brain stereotaxic coordinate showing the localization of the membrane of the microdialysis probe in the SCN. All animals with probes located in the SNC were included in this study. The effective sampling area for the microdialysis probes was approximately 1-2 mm around the membrane.

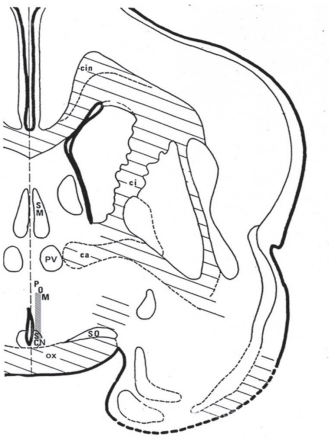

Figure 1: Schematic stereotaxic section from a rabbit brain showing the location of the membrane of the microdialysis probes in the SCN (hatched bar, membrane=20 mm shaft length and 2 mm length, drawn to scale). OX=optic chiasm, SCN=suprachiasmatic nucleus, SO=supraoptic nucleus, POM=preoptic nucleus, medial part, PV=paraventricular nucleus, SM=septal medial nucleus, cin=cingulum.

Neurochemical release in the SCN prior to, during and post parturition

The pregnant rabbits, exposed to the 16:8 h light-dark cycle, showed a higher GLU level in the SCN, prior to and following parturition, except during the actual birth, in comparison with animals exposed to the 12-h shifted photoperiod (Figure 2, Panels A and B). GLU concentration in the SCN was also significantly higher in estrous non-pregnant animals exposed to 16:8 h light-dark cycle photoperiod, compared to non-pregnant animals, subjected to the 12- h shifted photoperiod (Figure 3, Panels A and B). However, the GLU level was higher, but not significantly so, in all animals exposed to the normal photoperiod regardless of their physiological state (pregnant and non-pregnant). The rabbits (pregnant and non-pregnant), with the 12 h shifted photoperiod displayed a reduced GLU level in SCN.

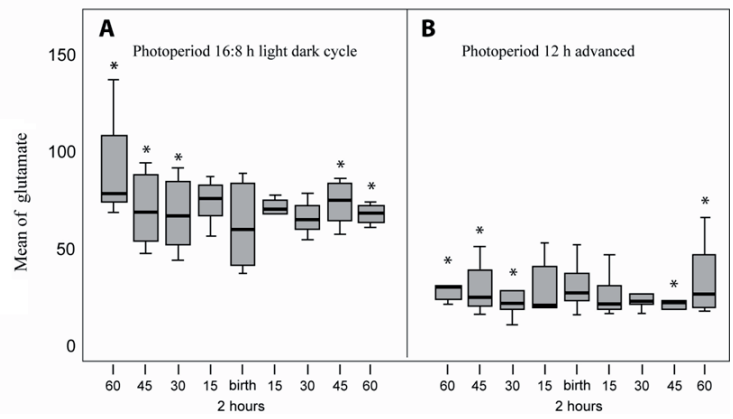

Figure 2: Mean glutamate concentration (nmol/20µl of cerebrospinal fluid) in pregnant rabbits. Panel A. Samples taken for 2-h (15 min intervals), prior to, during and after parturition in animals subjected to 16:8 h light-dark cycle. Panel B. Samples taken at similar intervals, but with a 12 h shifted photoperiod. Horizontal lines represent the 25th 50th (median), and 75th percentiles. Significance: **P*<0.009, **P*<0.04, **P*<0.02, **P*<0.009, and **P*<0.05, for −60, −45, −30, +45 and +60 minutes respect to birth when comparing both groups using the Mann-Whitney test.

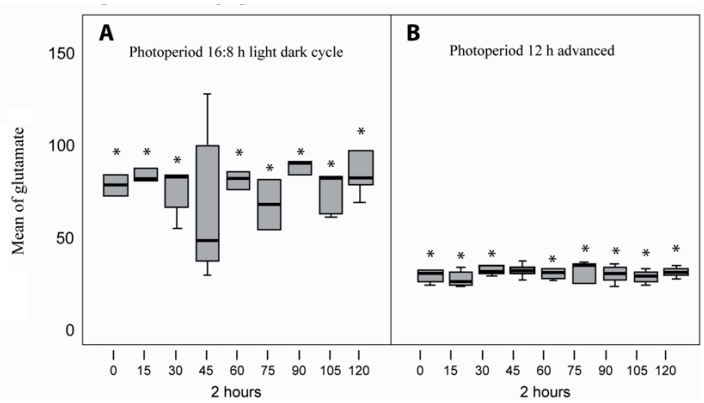

Figure 3: Mean glutamate concentration (nmol/20µl of cerebrospinal fluid) in estrous non-pregnant rabbits. Panel A. Samples taken for 2 h (15 min intervals), animals subjected to 16:8 h light-dark cycle. Panel B. Samples taken at similar intervals, but with the 12 h shifted photoperiod. Horizontal lines represent the 25th 50th (median), and 75th percentiles. Significance *P<0.009 for all the times controlled, except for 45 minutes (4th control), comparing the two groups using the Mann-Whitney test.

For each pregnant animal, we also compared the gestation length, the day of parturition, the hour and exact time of birth, the number of young rabbits per litter, and whether these were born alive (Table 1). The photoperiod seems to have an effect on the pregnant rabbits. Those exposed to a normal light photoperiod had a larger values for young per litter and parturition time generally lasted no longer than 15 min, being all the births on day 31 of gestation. Pregnant animals studied under the 12 h advanced photoperiod showed longer values for gestation period and parturition process (Table 1).

DISCUSSION

In this paper, we outline the findings on the GLU levels in the SCN from rabbits exposed to two different light photoperiods. These results confirm the prediction of a diurnal fluctuation in GLU levels, in animals exposed to a light period, and are consistent with extra cellular recordings and molecular studies (Cui *et al.*, 1997; Maciej *et al.*, 2000) of Syrian hamster SCN, which reflect the daily variation in SCN responses

Table 1: Gestation length, hour and time of birth and number of young rabbits per each animal.

Photoperiod	Gestation length (d)	Hour of Parturition	Born alive (Number)
16:8 h	31	11:05 - 11:15	3
	31	11:30 - 11:40	6
	31	09:10 - 09:15	4
	31	10:06 - 10:12	4
	31	12:00 - 12:10	7
12 h shift	31	16:40 - 17:00	3
	33	17:00	1
	32	20:00	1
	34	17:30	1
	32	17:50	1

(Ding *et al.*, 1994; Mikkelsen *et al.*, 1992). The GLU level was significantly higher for those rabbits exposed to the light phase regardless of their physiological state than for rabbits subjected to the 12 h shifted photoperiod. The pregnant animals exposed to the shifted photoperiod had a longer gestation period, prolonged parturition time, and a reduced litter size (Table 1).

The use of (Zolazepam chlorhydrate) during the mycrodialysis probing, did not affect the parturition process. However, we cannot rule out the possibility that it may have had some influence on neurochemical release in the SCN. Rabbits receiving this treatment during the last stage of pregnancy did not show any changes during the corresponding sampling period in comparison with samples from non-pregnant estrous animals.

In this study we revealed that the GLU level is significantly different in the SCN of pregnant animals exposed to light, prior to and following parturition as opposed to pregnant rabbits under the 12 h shifted photoperiod. Similar results were observed in the non-pregnant estrous animals exposed to the same photoperiods. Although no significant differences were observed between pregnant and estrous rabbits with regards to the GLU level of the SCN, light exerted a marked effect on the total litter size, gestation length, and time of parturition. It has been previously established that a 16:8 h light-dark cycle (natural period) seems to have a positive effect on reproduction (Rafay, 1992; Theau-Clement *et al.*, 1990; 2007). However, productivity in terms of number of young per litter and their viability has yielded contradictory results (Ducsay, 1996; Uzcategui and Johnston, 1992). With regards to our study, it is difficult to offer an interpretation of the results obtained from pregnant animals with a 12- h advanced photoperiod and the number of young rabbits litter size per parturition. As such, this will prove to be a worthwhile objective for further studies. In conclusion our results, although derived from a small number of female rabbits (in compliance as recommended by the Ethics Committee) suggest that light alters the GLU level in rabbit SCN. These variations could indicate an interaction between the photoperiod and the GLU level, and could have positively influenced the length of gestation, the number of young and the time of birth. These findings provide new insights into the diurnal regulation of neurotransmitters in the central processes underlying the reproduction processes and their dependence on the photoperiod. However, in order to confirm these results further studies, involving new biotechnologies and greater numbers of animals, are required. Further research will also help determine whether, in the long term, the stimulatory effect of the photoperiod and the GLU levels, in combination with other neurotransmitters and hypothalamic nuclei, are involved in the process responsible for triggering parturition in rabbits.

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