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Maternal Coordination of the Fetal Biological Clock in Utero

Abstract. *Deoxyglucose labeled with carbon-14 was used in studying the utilization of glucose in the suprachiasmatic nuclei of fetal rats. The results showed that an entrainable circadian clock is present in the suprachiasmatic nuclei during fetal development and that the maternal circadian system coordinates the phase of the fetal clock to environmental lighting conditions.*

Circadian rhythmicity of biological processes is the overt expression of an endogenous timekeeping mechanism (*1*). The rhythms are normally coordinated (entrained) to the 24-hour day by periodic environmental time cues, the daily alteration in light and darkness being one of the most potent entraining stimuli. Entrainment ensures a state of internal temporal order whereby the rhythms are expressed in proper relation to each other and to the 24-hour day. This helps to optimize the economy of biological systems and prepares an organism to foresee and to cope with alterations in the environment.

In mammals, the hypothalamic suprachiasmatic nuclei (SCN) appear to function as an endogenous pacemaker (biological clock). This concept was based originally on the results of lesion studies (*2*) and has been strengthened by the findings that the metabolic and electrical activities of the SCN vary on a circadian basis (*3*, *4*). Photic information for entrainment reaches the SCN from the retina via a direct retinohypothalamic pathway (*5*).

Most work on the mammalian circadian timing system has been done with adult rodents, and little effort has been directed at elucidating the development of this system. A major reason for this deficiency is that most circadian rhythms are not overtly expressed in rodents until the third week of life (*6*), making study of the activity of an entrainable circadian oscillator before that time difficult. An ingenious approach to this problem was pioneered by Deguchi (*7*), who used the phase (timing) of an overt rhythm monitored under constant conditions during the postnatal period to infer what had happened to the central oscillator underlying the rhythm at an earlier developmental stage. With this method, he provided data in the rat to suggest that the developing biological clock might be functional and entrainable by the mother during fetal life (*7*). However, the possibility that some aspect of the birth process itself starts and sets the timing of the developing clock cannot be readily excluded by this indirect approach (*8*). Proving that the fetal circadian clock functions before birth requires a method that could measure an intrinsic, functionally relevant property of the clock itself.

An autoradiographic technique in which ^{14}C-labeled deoxyglucose is injected intravenously allows for the simultaneous determination in vivo of the rates of glucose utilization of individual brain structures (*9*). Since brain structures are dependent on a continuous supply of glucose for energy (*10*) and since brain energy utilization and functional activity are closely linked (*11*), the amount of glucose utilized by an area reflects the overall functional activity of that area. Glucose utilization has provided an effective assay for oscillatory activity of the SCN in the adult rat (*3*). We applied this strategy to study the SCN in the fetal rat and now report that the fetal nuclei manifest a clear daily rhythm of glucose utilization and that the maternal circadian system coordinates the phase of the fetal clock to environmental lighting conditions (*12*).

In our first experiment, two pregnant albino rats were subjected to daily cycles of 12 hours of light and 12 hours of darkness, with lights on from 0700 to 1900 (designated LD) beginning early in pregnancy (*13*). On day 10 of gestation, each animal was outfitted with an intraatrial Silastic catheter (*14*). On day 19 of gestation, both pregnant rats were placed in and thereafter maintained in constant darkness. While the animals were in darkness, deoxyglucose (145 μCi/kg) (Amersham; specific activity 60 Ci/mole) was rapidly injected through the venous catheter. One rat was given the injection during her subjective night at 2300 hours on day 20 of gestation, and the other was given the injection during her subjective day at 1100 hours on day 21 of gestation; the injection times were,

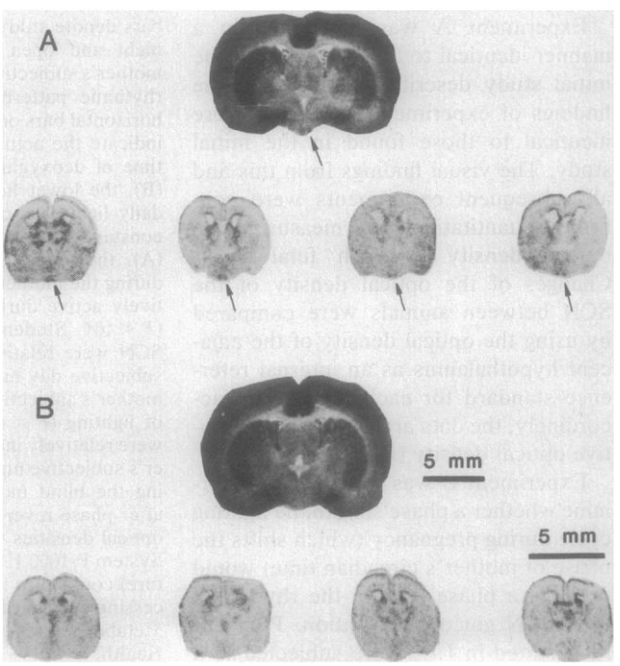

Fig. 1. Autoradiographs of coronal brain sections from mother and four of her fetuses after maternal injection during (A) subjective day and (B) subjective night. The metabolically active SCN appear as a pair of dark spots in the mother and fetuses during the subjective day (arrows), while the nuclei are relatively inactive and no longer visible during the night. The location of the SCN in the sections used to generate the autoradiographs in the lower panel was verified by cresyl violet staining.

respectively, 36 and 24 hours before the expected time of birth. At 45 minutes after deoxyglucose injection, both animals were decapitated and the abdomens were opened; the fetuses were then removed and decapitated. Maternal and fetal brains were frozen in cooled 2-methylbutane (−30° to −40°C). Frozen 20-μm coronal sections of brain were then cut on a cryostat, dried, and autoradiographed (*15*). After the autoradiographs were made, sections were stained with cresyl violet to determine the location of the SCN.

During the subjective day, the SCN were metabolically active and clearly visible as a pair of dark spots in the autoradiographs of the maternal brain and every fetal brain (eleven fetuses, six male and five female). In contrast, during the subjective night, the SCN were barely visible in the autoradiographs of the maternal brain and were not visible in any of the fetal brains (nine fetuses, seven male and two female). Thus, the fetal SCN manifest a clear, consistent rhythm of glucose utilization, and the fetal rhythm is synchronous with that in the mother.

We next performed three experiments to examine the role of the mother in coordinating the phase of the fetal clock. Unless otherwise specified, the methods for these studies were the same as those outlined above. Since the pattern of glucose utilization by the SCN was consistent among all the fetuses of one litter (either visible or not visible), we randomly chose four fetuses from each pregnant rat. For each of the deoxyglucose injection times, two pregnant rats were used, yielding eight fetal brains.

Experiment A was performed in a manner identical to that outlined for the initial study described above, and the findings of experiment A (Fig. 1) were identical to those found in the initial study. The visual findings from this and all subsequent experiments were confirmed quantitatively by measuring the optical density of each fetal SCN. Changes of the optical density of the SCN between animals were compared by using the optical density of the adjacent hypothalamus as an internal reference standard for each fetal brain; accordingly, the data are expressed as relative optical density (Fig. 2A).

Experiment B was designed to determine whether a phase shift in the lighting cycle during pregnancy (which shifts the phase of mother's circadian time) would produce a phase shift in the rhythm of fetal SCN glucose utilization. Pregnant rats, mated in LD, were subjected to a progressive phase delay in the lighting cycle until a reversed light cycle (designated DL, with lights on from 1900 to 0700) was attained on day 6 of gestation (*16*). The animals were kept in DL until day 19 of gestation when they were placed in and thereafter maintained in constant darkness. One group of pregnant rats was then given the deoxyglucose injection during their subjective day, now at 2300 hours on day 20 of gestation, and the other group was given the injection during their subjective night, now at 1100 hours on day 21 of gestation. The SCN were still clearly visible in each fetal autoradiograph obtained during subjective day and not visible in those obtained during subjective night (Fig. 2B). Thus environmental lighting, acting either alone or through the maternal circadian system, coordinates the timing of the SCN during fetal life.

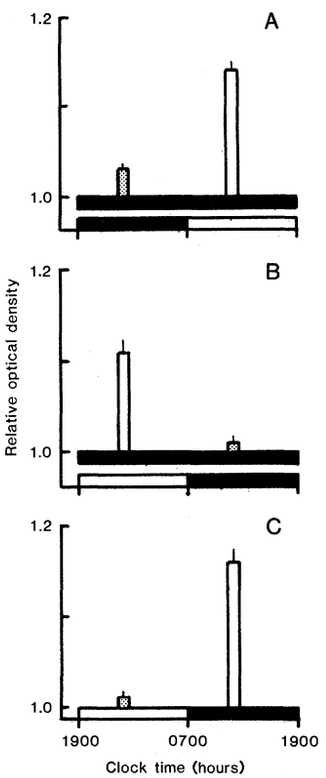

Fig. 2. Relative optical densities (OD) of fetal SCN (optical density of the SCN divided by the optical density of the adjacent hypothalamus) under various experimental conditions. The height of each vertical bar is the mean ± standard error of the relative optical density of the SCN of eight fetal brains; stippled bars denote study during mother's subjective night and open bars denote study during mother's subjective day, as determined by her rhythmic pattern of drinking activity. The horizontal bars on which the vertical bars rest indicate the actual lighting conditions at the time of deoxyglucose injections. In (A) and (B), the lower horizontal bars represent the daily light-dark cycles prior to placement in constant darkness on day 19 of gestation. In (A), the fetal SCN were relatively inactive during the mother's subjective night and relatively active during mother's subjective day ($P < .01$, Student's t-test). In (B), the fetal SCN were relatively active during mother's subjective day and relatively inactive during mother's subjective night after phase reversal of lighting ($P < .01$). In (C), the fetal SCN were relatively inactive during the blind mother's subjective night and relatively active during the blind mother's subjective day even after phase reversal of lighting ($P < .01$). All optical densities were read on the Photoscan System P-1000 HS densitometer (50-μm aperture) coupled to the computerized image processing system of the Laboratory of Cerebral Metabolism, National Institute of Mental Health.

These two possibilities were examined in experiment C. Pregnant rats were blinded on the first day of gestation after having been entrained to LD (*17*). The animals were then exposed to the phase change in lighting, described for experiment B, until DL was achieved; the animals were thereafter kept in DL. One group of rats was given the deoxyglucose injection in the light during subjective night at 2300 hours on day 20 of gestation, and the other group was given the injection in the dark during subjective day at 1100 hours on day 21 of gestation (*18*). The fetal SCN were not visible in the autoradiographs obtained after the injection in light (during the mother's subjective night), but they were visible in those obtained after the injection in the dark (during the mother's subjective day) (Fig. 2C). Hence, the metabolic activity of the fetal SCN is out of phase with that expected if environmental lighting directly influences the timing of the fetal clock. Rather, the fetal rhythm is synchronous with the circadian time of the blind mothers.

Our results indicate that at a time in development well before the maturation of the neural mechanisms necessary for both the photic entrainment and overt expression of circadian rhythms (*19*), the mother acts as a transducer between the environment and the fetal brain, coordinating the phase of the developing biological clock to her own clock time which, in turn, is entrained by ambient lighting. This form of maternal communication appears to have evolved to prepare the developing mammal for entry into the external environment. As the mechanisms necessary for the expression of circadian rhythms mature, maternal coordination would ensure that the developing rhythms are expressed in appropriate temporal relation to each other and to the 24-hour day. Without maternal coordination, circadian rhythms would develop uncoordinated until the

time that direct contact with environmental lighting acted to synchronize the phase of the rhythms. This period of disorganization might render the already vulnerable neonate more susceptible to various insults that could influence survival.

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12. Glucose utilization by the SCN has been studied by the deoxyglucose technique in the developing rat [J. L. Fuchs and R. Y. Moore, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1204 (1980)]. Although these investigators found a clear day-night variation of SCN glucose utilization during the first day of postnatal life, they were unable to detect a rhythm in the fetus (examined at day 20 of gestation).
13. Timed pregnant Sprague-Dawley rats (Zivic-Miller, Allison Park, Pa.) were shipped to us within the first 4 days after insemination; day 0 of gestation was designated as the day the rats showed a positive reaction for sperm. Each rat was housed singly in a clear plastic cage. Each cage was wired to a drinkometer relay so that the daily profiles of drinking behavior were monitored for each pregnant rat. The light portion of the lighting cycles provided 600 lux of light at the midcage level. Rat food and water were freely available, and the time of day of routine care was varied.
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16. Maternal drinking behavior rapidly adjusted to the phase shifts and it was completely entrained to DL by day 9 of gestation.
17. Animals anesthetized with ether were blinded by bilateral orbital enucleation.
18. The rhythmic onset of drinking in the blind rats was delayed only about 1 hour when deoxyglucose was administered (3 weeks after they were blinded), compared to that in sighted animals maintained in LD throughout pregnancy.
19. The retinohypothalamic pathway does not innervate the SCN until postnatal day 3 to 4 [M. Felong, *Anat. Rec.* **184**, 400 (1976); B. Stanfield and W. M. Cowan, *Brain Res.* **104**, 129 (1976)].
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Hormonal Control of Sexual Differentiation: Changes in Electric Organ Discharge Waveform

Abstract. *Males and females of some mormyrid electric fishes generate electrical pulses that differ in waveform and duration. For one such species, testosterone or dihydrotestosterone induces females and immature males to produce the mature male electric organ discharge which is two times the duration of the female or immature discharge. Estradiol has only a weak effect. For a second species where males and females have similar electric organ discharges, testosterone produces no effect. The data suggest that androgens affect the electric organ itself.*

Androgen- and estrogen-concentrating cells occur in gonadal tissues and in skeletal muscle and brain structures implicated in the control of vertebrate sexual behavior (*1*). Gonadal hormones can affect a variety of chemical and morphological processes ranging from acetylcholinesterase activity of nerve and muscle to dendritic growth of central neurons (*2*). We now propose that steroid hormones can modulate the development of action potentials of some electrically excitable membranes (*3, 4*).

For some mormyrid electric fishes from Africa, the electric organ discharges (EOD's) of males and females differ in waveform and duration (Fig. 1, d to f) (*5*). The electric organ, situated in the caudal peduncle (see Fig. 1, a and b), is derived from myomeres and includes four columns of serially stacked plates or "electrocytes" oriented in the transverse plane. For each electrocyte, an anterior or posterior face has rootlike extensions that join into a major stalk that is innervated by special spinal motoneurons, the "electromotoneurons" (*6, 7*). The EOD is elicited by a burst of three spikes in the electromotoneurons, which in turn are excited by a descending medullary volley (*7*). The membrane of each face and stalk produces an action

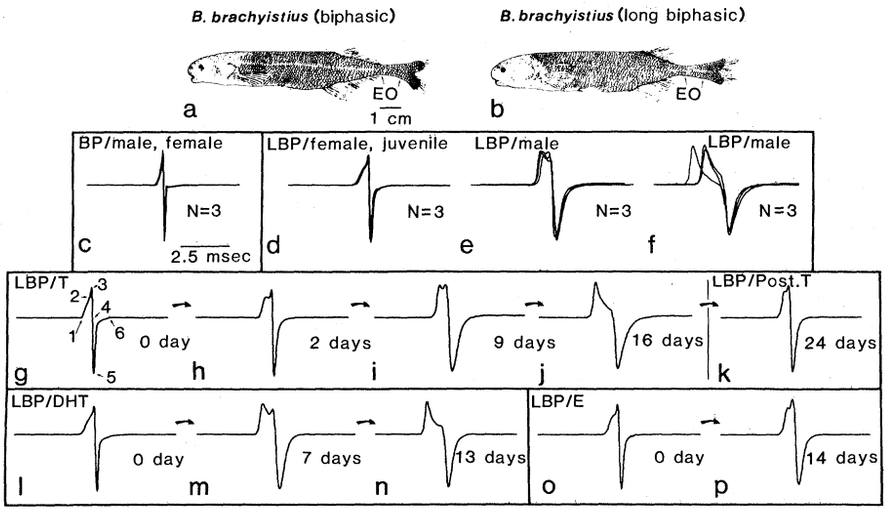

Fig. 1. (a and b) The two species of electric fish in this study were *Brienomyrus brachyistius* (biphasic) (BP) and *B. brachyistius* (long biphasic) (LBP); the electric organ (*EO*) is in the tail. (c to p) Oscilloscope records of electric organ discharge (EOD) waveforms scaled to the same peak-to-peak amplitude. (c) *B. brachyistius* (BP) males and females have similar EOD's (three superimposed individual EOD's). The EOD of juvenile and female *B. brachyistius* (LBP) are similar (d) but differ in form and duration from males (e and f). (g to j) Testosterone (*T*) added to the water of a *B. brachyistius* (LBP) female induces the male EOD for 16 days; the EOD reverts to a female type (k) after the fish is returned to freshwater for 24 days. (l to n) A pellet of DHT implanted in a gonadectomized female also induces a male EOD. (o and p) An estradiol (*E*) pellet implanted in an immature male has minimal effects on the EOD.