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Research report

Metabolic activation of amygdala, lateral septum and accumbens circuits during food anticipatory behavior

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HIGHLIGHTS

- We used cytochrome oxidase histochemistry to study brain metabolic circadian rhythms.
- Neural rhythms were studied in rabbit pups entrained to circadian feeding.
- Extended amygdala, lateral septum and accumbens core peaked during food anticipation.
- Accumbens shell, caudate, putamen and cortical amygdala peaked after feeding.
- Temporal conditioning circuits may contribute to food anticipatory activity.

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ABSTRACT

When food is restricted to a brief fixed period every day, animals show an increase in temperature, corticosterone concentration and locomotor activity for 2–3 h before feeding time, termed food anticipatory activity. Mechanisms and neuroanatomical circuits responsible for food anticipatory activity remain unclear, and may involve both oscillators and networks related to temporal conditioning. Rabbit pups are nursed once-a-day so they represent a natural model of circadian food anticipatory activity. Food anticipatory behavior in pups may be associated with neural circuits that temporally anticipate feeding, while the nursing event may produce consummatory effects. Therefore, we used New Zealand white rabbit pups entrained to circadian feeding to investigate the hypothesis that structures related to reward expectation and conditioned emotional responses would show a metabolic rhythm anticipatory of the nursing event, different from that shown by structures related to reward delivery. Quantitative cytochrome oxidase histochemistry was used to measure regional brain metabolic activity at eight different times during the day. We found that neural metabolism peaked before nursing, during food anticipatory behavior, in nuclei of the extended amygdala (basolateral, medial and central nuclei, bed nucleus of the stria terminalis), lateral septum and *accumbens* core. After pups were fed, however, maximal metabolic activity was expressed in the *accumbens* shell, caudate, putamen and cortical amygdala. Neural and behavioral activation persisted when animals were fasted by two cycles, at the time of expected nursing. These findings suggest that metabolic activation of amygdala-septal-accumbens circuits involved in temporal conditioning may contribute to food anticipatory activity.

Abbreviations: CO, cytochrome oxidase; FAA, food anticipatory activity; OD, optical density; PD, postnatal day; ROD, relative optical density; ZT, zeitgeber time.

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1. Introduction

The hypothalamic suprachiasmatic nucleus (SCN) is the master circadian pacemaker in mammals due to its capacity for coordinating circadian oscillators in other brain regions and peripheral tissues, thus regulating physiological and behavioral rhythms [1]. These circadian rhythms are sustained by molecular autoregulatory feedback loops that oscillate with a ~24 h period [2]; however, molecular, physiological and behavioral rhythms also can be entrained by daily cycles of food availability, which are not abolished by SCN lesions [3]. When animals are entrained to a single and brief feeding period each day, they show increased physiological parameters and locomotor behavior, named food anticipatory activity (FAA), 2–3 h prior to scheduled feeding. FAA exhibits properties of a circadian clock-controlled process and can be considered the output of food-entrainable circadian oscillators [4]; nonetheless, all the neural components and mechanisms responsible for FAA are not well understood. The role of particular neural structures involved in FAA has been broadly investigated, but the fact that lesion approaches have not yet revealed a critical role for any of the studied regions suggest that the anatomical substrates generating FAA could involve multiple oscillators [5] as well as neural networks related to memories formed by temporal conditioning [6].

A natural model to study the mechanisms underlying food entrainment and FAA is the rabbit pup. Lactation behavior in the rabbit is the most restrictive maternal care pattern exhibited among mammals, since rabbit pups are nursed only once a day for just 2–4 min every 24 h in constant darkness. Pups are born altricial, *i.e.*, furless, with eyes and auditory canals closed and unable to control their body temperature, so they remain huddled together inside the nest for most of the 24 h cycle. However, 2–3 h before the scheduled nursing, when pups have an almost empty stomach, they display FAA: their body temperature and locomotor activity increase, remaining very active until the dam's visit [7]. These feeding characteristics demand a much greater challenge in the rabbit pup than is required in other newborn species, since a failure to be awake and ready for suckling could lead to a loss of food ingestion and starvation.

In rabbit pups, as well as food-entrained adult rodents, FAA is accompanied by an increase in plasma corticosterone concentration in anticipation to the daily meal [8]. Corticosterone levels continue high for a few hours after feeding and then decline until the next cycle. When food is omitted, corticosterone continues peaking at the programmed feeding time, but notably with higher levels compared to non-fasted subjects [8,9]. This rhythmic pattern of daily plasma corticosterone during the first two weeks of life of the rabbit clearly contrasts with that of neonatal rodents, which show very low and stable levels of corticosterone, and a reduced capacity to secrete it in response to several stimuli – stress hyporesponsive period – [10,11]. This rhythmic increase in corticosterone may contribute to prepare the pups for nursing, both in terms of catabolizing energy stores and behaviorally, increasing the adaptive capacity of the animal to a demanding situation of stiff competition for milk among siblings since the first postnatal days [12]. The vital importance of being awake in anticipation of the brief nursing, together with the high competitive behavior among the pups for the nipples and the high levels of corticosterone suggest that rabbit pups need to be alert in a motivationally aroused state before the nursing bout, *i.e.*, when FAA is expressed. This emotional state may prepare the pups to develop adequate strategies for improving feeding opportunities. Therefore, we hypothesized that in the rabbit pupís brain, the metabolic activity of amygdala circuits triggering alertness and conditioned emotional responses would be maximal during FAA in preparation for feeding events, and that the 2–4 min interaction with the doe provides an appeasing stimulus resulting in the decrease of their metabolic activity after nursing. Because of the pleasant components involved in feeding, we also expected that other structures more related to consummatory reward would be entrained to nursing with maximal metabolic activity after feeding.

To test our hypothesis, we measured neural metabolic activity with the cytochrome oxidase (CO) histochemistry method, as previously reported [13]. CO is the terminal enzyme of the electron transport chain in the mitochondria, which catalyzes the oxidation of cytochrome *c* and the reduction of molecular oxygen. Changes in the physiological activity of neurons induce parallel changes in CO histochemical activity in developing and adult brains [14]. Hence CO histochemistry has been used as a reliable method for measuring regional brain metabolism under different behavioral conditions [15], including circadian [16,17] and conditioning learning studies [18,19]. CO activity can be measured optically using diaminobenzidine (DAB) as the electron donor to reduce cytochrome *c* [14]. Under specific incubation conditions, a linear relationship can be found between the optical density (OD) of the reaction product – oxidized DAB – and CO enzymatic activity [20]. Therefore, we quantified the OD of the histochemical reaction (oxidized DAB) as a measure of metabolic activity in neural structures mainly related to alertness and emotional arousal: basolateral (BLA), central (CeA), medial (MeA) and cortical (CoA) nuclei of the amygdala, bed nucleus of the stria terminalis (BNST, dorsal portion in reference to the anterior commissure), dorsal, intermediate and ventral parts of the lateral septal nucleus (LS), and in structures highly related to rewarding processes: nucleus *accumbens* (NA), caudate (Cau) and putamen (Pu). The OD of these areas was measured in litters nursed at 1000 h from postnatal day (PD) 1–7. At PD7, when all litters showed clear FAA, rabbit pups were euthanized at different time points to cover a complete 24 h cycle (Nursed Group). In order to explore if the neural oscillations are maintained even without the nursing event (zeitgeber), a second group was included: litters were nursed at 1000 h from PD1 to 7, followed by two cycles of food deprivation, and brains were collected and analyzed along PD9 (Fasted Group). Finally, the metabolic activity profile of brain structures of each group was temporally correlated with its respective pattern of circadian locomotor behavior.

2. Material and methods

2.1. Animals and housing

New Zealand white rabbits (*Oryctolagus cuniculus*) were reared in our animal facility. After mated, pregnant females were housed individually in stainless steel cages with adequate ventilation in an artificial 12/12 h light/dark cycle (lights on at 0700 h) at 22 °C ± 2. Water and food pellets (Purina rabbit chow) were offered *ad libitum*. Each cage consisted of two compartments: one for the mother, exposed to the light/dark cycle, and the other for the nest, which was maintained in constant darkness. Between the compartments there was a tunnel with two opposite sliding doors to avoid light entrance when the doe visited the nest. To determine behavioral entrainment and FAA, 24 h locomotor activity of each litter was monitored by infrared detectors sensitive to pupsí movements, which were located on the ceiling of each nest [13,21]. In the last week of pregnancy, females had free access to the nest compartment, where they built their nest with provided straw and fur from their own belly. On the day of delivery, each litter was adjusted to eight pups; at the same time, mothers were relocated to a contiguous room with the same room conditions as described above, to avoid litters from being disturbed by their own dam.

Animal care and experimental procedures were carried out in accordance with the procedures of the National Guide for the