Prenatal dietary choline supplementation modulates long-term memory development in rat offspring

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ABSTRACT

The development of an organism is modulated by multiple factors, with genes and diet being examples of such factors. Previous studies on preclinical models have shown that giving supplemental choline – an essential nutrient to mammals – during the embryonic period improves performance on memory tasks during adulthood. However, the effects of an early intervention on the development of cognitive functions in the immature brain have not been widely studied. In addition, it has been well established that short-term memory in rats emerges at an earlier stage (14–15 days postnatal) than long-term memory (around 30 post-natal). The aim of this work was to examine the effect of prenatal dietary choline supplementation on long-term memory development in rats. In order to assess long-term memory, we used an object-recognition task, which evaluates the ability to recall a previously presented stimulus. Pregnant rats were fed with the diets AIN 76-A standard (1.1 g choline/Kg food) or supplemented (5 g choline/Kg food) between embryonic days (E) 12 and E18. On the first post-natal day (PN 0), male offspring of the rats fed with the supplemented and standard diet were cross-fostered to rat dams fed a standard diet during pregnancy and tested at the age of PN21-22 or PN29-31 applying 24-hour retention tests. The supplemented animals spent less time exploring the familiar object after a 24-hour retention interval, an effect that was observed in both the group tested at PN21-22 days of age and that tested at PN29-31 days. The non-supplemented rats only showed this effect in the group tested at PN29-31 days. These results suggest that prenatal supplementation with choline accelerates the development of long-term memory in rats.

Introduction

During the early post-natal period, the brain undergoes rapid growth, with different areas of the brain developing at different speeds. The oldest phylogenetic areas such as the corpus striatum and motor area, emerge functionally earlier than other areas associated with superior cognitive processes such as the hippocampus and frontal cortex [1]. This process occurs in all mammal species with similar developmental trends, including human, primates, and rats [2]. The brain, in its early stages of development, is highly sensitive and experience provokes lasting changes in the organism. Development is considered to be a dynamic process through which both the structure and biological functionality of an organism is defined through complex interactions between the genes and the context [3]. Moreover, diet is considered to be a determining factor in this developmental process [4,5], where nutrients carrying methyl groups, such as choline, are related to lifelong changes in gene expression [6].

Several studies using rodents have shown that prenatal supplementation with choline, which is an essential nutrient for human and non-human animals [7,8], facilitates cognitive functions during adulthood [9]. Choline supplementation during embryonic days E11 to E17 significantly improves spatial and temporal memory as well as attention [10,11]; for a review see 12 and spatial memory when the manipulation is carried out on PN 16-30. Likewise, it has been observed that these effects remain during the life of the animal, protecting them against cognitive deficits related to aging [13,14].

The novel object recognition (NOR) task has long been employed to study recognition memory in rodents. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. In the standard NOR procedure rats are initially exposed to two identical objects for a given time (Familiarisation phase), whereupon one of the familiar objects is removed and, after a retention interval, the rat is given a test with both the familiar object and a novel object (Test phase) and the time that the animal spends exploring each one is recorded.
Because rats prefer to explore the new stimulus rather than the familiar object, it is inferred that they remember the previously known stimulus. Hence, NOR is a paradigm where the spontaneous preference for novelty is used as an index of retention in rodents. The NOR test has certain advantages such as the fact that spontaneous preference is common in mammals, and this allows for interspecies comparisons; it is not based on positive or negative reinforcers such as food or electric shocks; and it is not associated with high levels of stress or physical activity [15]. Furthermore, depending on the retention interval between the familiarisation and test phase, we can evaluate both short (e.g. seconds or minutes) and long-term (e.g. hours or days) memory. Consequently, this test can be usefully applied with neuroscientific approaches such as lesions, immediate-early gene imaging, or pharmacological studies, leading to a better understanding of memory in rats [16].

The relevance of the cholinergic system in the NOR task has been widely demonstrated with rats. For instance, an increase in levels of synaptic acetylcholine (ACh) caused by systemic treatment with inhibitors of the enzyme that degrades the neurotransmitter acetylcholinesterase (AChE) had the effect of reducing the deficit in NOR that is otherwise observed in aged rats [17,18]. In contrast, the selective destruction of cholinergic neurons in the perirhinal cortex with the saporin immunotoxin 192 IgG, has been shown to cause a deterioration in NOR performance [19]. Further, the use of cholinergic receptor antagonists and agonists has pointed to the importance of ACh in the object recognition task. For instance, systemic administration [20] and to the perirhinal cortex [21] of scopolamine, a cholinergic muscarinic receptor antagonist, has been shown to impair discrimination between a novel and familiar object on a NOR task. Additionally, other studies have found that an intraperitoneal injection of a nicotinic agonist before or after the familiarisation phase, or prior to the test phase, resulted in better NOR performance [22]. More specifically, nicotine injections administered to either the hippocampus or the perirhinal cortex prior to the familiarisation phase improved performance on a NOR task [23]. These latter findings imply a role for the nicotinic receptors in the acquisition, consolidation, and retrieval of object information.

To date, studies of supplementation with prenatal choline have focused mainly on evaluating the long-term effects on different cognitive functions during adulthood. However, to the best of our knowledge, the effects of an early intervention on the development of cognitive functions in the immature brain have not been widely studied, except for the work conducted by Mellott et al. [24]. These authors showed that prenatal choline supplementation advanced the emergence of rats ability to use spatial cues to navigate in a water maze, demonstrating for the first time an acceleration of hippocampal function and development following prenatal choline supplementation. Cognitive skills emerge sequentially in an order of increasing complexity, whilst simpler skills emerge before those of a higher order, in accord with the development of the neural systems that are involved in the processes [25]. For instance, short-term memory emerges before long-term memory. The latter depends on the maturity of the hippocampal system along with the motor and sensory systems. In fact, Rudy & Morledge [26] found that the short-term memory representation of a context (30 s) that mediates conditioned fear emerges in 18-day-old rats, whilst long-term memory (24 h) was only observed in 23–32-day-old rats. Developmental studies suggest that cognitive skills emerge once the brain system that supports them (or connections to such systems) is mature [27]. The ability to demonstrate basic cognitive abilities such as memory in a prescribed task requires the sensory systems, which develop sequentially, and in the rat, are fully functional around the second week of life. It has been found, using classical conditioning paradigms, that the capacity to associate stimuli depends on the maturity of the sensory modality involved. The smell, taste, or somatosensory [28,29] auditory [30], and visual systems [31,32] typically develop around PN12 and PN15. Such skills are evident when the test is conducted shortly after acquisition (short-term or procedural memory). For its part, long-term memory usually emerges around 30 days after birth in rats. Findings relevant to this issue come from studies showing that it is possible to establish a conditioned response to a visual [33] or contextual [26] stimulus with conditioning procedures in PN16-PN18 rats. However, when the retention tests are conducted after long periods of time (24 h or a week), the conditioned response (CR) is only observed when the rats are conditioned at around 30 days old [34–36].

The aim of the current study was to assess whether choline supplementation during gestation days 12–18 affects the development of long-term object recognition memory in rats, which, as mentioned, emerges around PN30 for many visuo-spatial tasks. Given our previous results, which show that prenatal choline supplementation enhances performance on NOR in adult rats, we hypothesised that it could also facilitate the development of long-term object recognition memory. For this purpose, PN21–22 pups, whose sensorial systems (for
example, the visual system) are fully mature, or PN29-31 pups from rats that were fed a supplemented or standard diet between E12-E18, were tested on a NOR task with a retention interval of 24 h between the familiarisation and test phases.

**Materials and methods**

**Subjects and diet**

A chronogram of the treatment and the experimental dietary groups, supplemented (SUP) and control (CTL), as well as the behavioural training period is shown in [Figure 1](#). Twelve pregnant Wistar rats were fed with a basal purified modified diet AIN 76-A. The rats were individually housed in cages (54 × 33 × 18 cm) with a room temperature between 22 and 23 °C and were maintained on a light/dark 12 h cycle. Water and food were available *ad libitum* during pregnancy.

On the afternoon of day E-11, the rats were divided into two groups and fed with the diets AIN 76-A standard (CTL, n = 6) or supplemented (SUP, n = 6), which contained 1.1 g/Kg choline chloride or 5 g/Kg choline chloride respectively. The dietary treatment continued until day E18. This concentration represents a 4.5-fold increase in choline compared with the standard diet (1.1 g/Kg) and this increase, as demonstrated in Meck and Williams' laboratory [12], enhances cognitive performance in rodents when they are prenatally supplemented during E12-18. Food and water were available *ad libitum*, and the food was weighed during the dietary manipulation to determine daily consumption.

After the intervention ended on E18, the dams were fed AIN 76-A (standard diet). On the day of birth (PN0), 32 male pups were cross-fostered by the CTL dams to control for differences in maternal care. The female pups were then used to carry out other studies in our laboratories. Each foster dam (n = 4) raised a litter of 8 non-biologically related pups (4 CTL and 4 SUP). On PN21 (weaning), the offspring were assigned to cages based on their experimental group membership (SUP or CTL), with 4 from the same experimental group to each cage. Cages were randomly assigned to be tested in the PN21-22 or the PN29-31 conditions, resulting in 8 CTL and 8 SUP animals tested in each condition on the NOR task. Two rats were excluded from the study (PN21-22/CTL y PN29-31/SUP) due to the lack of exploration during the familiarisation phase. After the test, the animals were euthanized using an overdose of anaesthesia (>1.5 mL equithesin/kg body weight, i.p.) followed by cervical dislocation. All the procedures in this study were approved by the Ethics Committee for Animal Research of our University (procedures 93-CEEA-OH-2015) and were conducted in compliance with the European Council directive 86/609/EEC and the Spanish law (R.D. 53/2013).

**Apparatus**

The objects used in the NOR task were two jars that differed in shape. One of the objects was a small rounded cup (6.5 cm in height by 6.8 cm wide) with a handle, while the other object was a small elongated jug (8.8 cm in length by 5.7 cm wide) without a handle. The exploration box was adapted to the age (size) of the pups in order to facilitate exploration (32 × 52 × 30 cm). The boxes were wrapped with black lining to avoid any distraction in the testing room, and the floors of the boxes were covered with sawdust. The objects were fixed to the floor of the boxes with Velcro (fast opening and closing system) in the corresponding location to avoid any movement of the objects during the task. A camera was positioned above the box to record the sessions. The box area was lit with a lamp in order to limit access to contextual information from the laboratory room.

![Figure 1](#). Chronogram of the experimental procedure. The dietary manipulation was applied on gestation day 12 until Day 18 (E12-18). On the first postnatal day (PN0), cross fostering was carried out. The behavioural procedure was conducted with two groups at different ages, PN21-22 or PN29-31.
**Behavioural procedure**

The trials began when the rats were at PN21-22 or PN29-31. The procedure involved three phases; habituation to the box, familiarisation, and the object recognition memory test. The animals received three habituation sessions during which they were allowed to freely explore the empty box for 10 min, and then, they were placed in the laboratory room. The first two habituation sessions were carried out on the same day, one in the morning (9 am) and one in the afternoon (2 pm). The third session took place on the morning of the next day (9 am). On the following morning at the same time, the familiarisation session began in which the animals were allowed to freely explore two identical objects placed in the box for a period of 5 min. For half the subjects in each group, these were the elongated jugs and for the other half these were the rounded cups. We conducted the test session 24 h later. For this test, one of the objects from the familiarisation phase was replaced by a novel object (elongated jugs or rounded cups). For half the subjects this was the object on the left, and for the other half this was the object on the right. The subject was exposed to the two objects for 3 min. Exploration was defined as the contact time with the objects (when the nose was 2 cm away from the object and there were movements of the vibrissae).

**Statistical analyses**

In order to analyse the intake of the rats during the dietary treatment in the E12-E18 period, we conducted a 2 (Diet: SUP, CTL) × 7 (Day: E12 to E18) mixed ANOVA with diet as the between-subjects factor and day as the within-subject factor. Multiple comparisons were used to compare the mean consumption of the rats between E12 and E18 during pregnancy. The Bonferroni correction was applied to correct the alpha inflation. We also analysed object exploration time during the familiarisation and test phases by means of a 2 (Age: PN22, PN31) × 2 (Diet: SUP, CTL) × 2 (Object: Novel, Familiar) mixed ANOVA with age and diet as between-subject factors and object as the within-subject factor. The interactions between factors were explored with simple effects analyses. A significance level of $p < 0.05$ was adopted for all statistical analyses.

**Results**

During the dietary treatment (E12-E18 period), an increase in the intake of the rats between days was observed in both groups, SUP and CTL. The mixed ANOVA with Diet (SUP vs CTL) as the between-subjects factor and Day (from E12 until E18) as the within-subject factor revealed a significant effect of day, $(F(6,60) = 15.82, p < .05)$. The main effect of diet was not significant $(F(1,10) = 1.01, p > .05)$, and neither was the interaction between Diet and Day $(F < 1)$ (6,60). Multiple comparisons with Bonferroni correction revealed that there was a significantly higher intake during the last two days of the manipulation, E17-E18, compared with consumption on days E12-15. The time spent exploring the objects during the familiarisation phase is displayed in Figure 2. Both objects, Familiar 1 and Familiar 2, were explored equally. A mixed ANOVA with Age (PN21-22 vs PN29-31) and Diet (SUP vs CTL) as between-subject factors and Object (Familiar 1 vs Familiar 2) as the within-subject factor, revealed no significant differences, highest $F(1, 26) = 1.35, p = 0.265$.

The time spent exploring the familiar and novel object during the test phase for each age group, PN21-22 or PN29-31, for the dietary treatments (SUP or CTL) are shown in Figure 3. Supplemented animals showed better recognition (less exploration) of the familiar object.

![Figure 2](image-url)  
**Figure 2.** Familiarisation phase. Exploration time means (±SEM) for each identical object (Familiar 1 and Familiar 2) during the familiarisation phase for the groups (PN21-22 and PN29-31) with different dietary treatments (Control and Supplemented).
compared with those that were fed with the standard diet for the age groups PN21-22. In contrast, for the age group PN29-31, animals in both Group SUP and CTL recognised the familiar object. These impressions were confirmed by conducting a mixed ANOVA with Age (PN21-22, PN29-31) and Diet (SUP, CTL) as between-subject factors and Object (familiar, novel) as the within-subject factor, which revealed a main effect of object ($F(1, 26) = 27.98, p < .05$), a significant interaction between Object and Diet ($F(1, 26) = 6.67, p < .05$) and a significant 3-way interaction between Object, Diet, and Age ($F(1, 26) = 5.25, p < .05$). No other main effect or interaction was significant, with the highest $F(1, 26) = 2, p > .05$. A simple effects analysis for each of the age groups revealed a main effect of object for Group PN21-22 ($F(1,13) = 7.36, p < .05$) and a significant interaction between Object and Diet ($F(1, 13) = 11.65, p < .05$), whilst the main effect of Diet was not significant ($F < 1$). The analysis of the interaction revealed significant differences between the familiar and novel object in the SUP Group ($F(1, 13) = 18.10, p < .05$) but not in the CTL Group ($F < 1$). For Group PN29-31 the main effect of object was significant ($F(1, 13) = 22.92, p < .05$) but not the main effect of Diet or the interaction between Object and Diet ($F(s) < 1$).

These results show that those animals supplemented with choline were capable of recognising the object after a long retention interval at an early (PN21) age compared with non-supplemented animals, for whom this ability was evident when tested a week later.

**Discussion**

We observed that prenatal choline supplemented rats had good 24 h memory retention at 21–22 days of age in contrast with rats fed the control diet that show 24 h retention of the object when the test was conducted a week later at 29–31 days of age. This result could be taken to suggest that prenatal choline availability accelerates the development of long-term memory, at least when expressed as performance on an object recognition task. In contrast, both of the older aged groups (PN29-31) that were in the SUP or CTL groups, showed a preference for the novel object without any significant differences between them (Figure 3).

Object recognition is one of the first mnemonic abilities observed in rodents. Krüger et al. [37] found that the ability to detect the presence of a novel object or to recognise a familiar one emerges once the maturation of the visual, tactile, and motor systems has been reached, that is, for rats at the PN14-15 age. According to this hypothesis, ontogenetic studies using an object recognition task have found that PN17 [38], PN18 [39], and PN20-23 rats [40,41] are capable of recognising a familiar object between 1 min and 1 h after the familiarisation phase. However, there are a limited number of studies examining the ontogeny of long-term memory using an object recognition memory task. The only available results have shown that the expression of long-term memory, as measured by NOR, emerges on PN29-40 rats [40], which requires the hippocampal circuit to have reached maturity [42]. Consistent with these results, in the present study PN21-22 non-supplemented rats (Group CTL) failed to recognise the familiar object after a long retention interval (24 h), and only those animals that were tested a week later could perform the task correctly (Figure 3). Similarly, using a place recognition memory task, PN21 rats spent more time exploring objects that had been moved to a location that was different to that used for familiarisation after a 5-min retention interval, whereas only rats that were a week older were able to retain information about the place where
the object had been presented 24 h prior to the test [37]. Several mechanisms may be considered to account for the effects of early life choline. As a multifunctional micronutrient, choline is a methyl group donor in the metabolic pathway of monocholines and phospholipids, phosphatidylcholine (PC) and the acetylcholine neurotransmitter. Given its functions, the behavioural potentiation caused by prenatal dietary choline has been associated with epigenetic changes in the pattern of DNA methylation [43] and the metabolism of the cholinergic system through an increase in the choline reserves as membrane phosphatidylcholine in the brain [44]. It has also been associated with the facilitation of the synthesis and release of acetylcholine [45,46], which is a neurotransmitter involved in memory processes [47] and attention [48].

A relevant contribution of the present study is that it demonstrates that an early intervention, i.e., a dietary choline manipulation, is able to advance the manifestation of cognitive processes. The results of previous studies support the possibility of modifying the functional development of the hippocampus. In fact, the increased depolarisation time of the AMPA glutamatergic receptors in the hippocampus, induced by a positive allosteric modulator [49], advanced the emergence of spatial navigation (dependent on the hippocampus) in PN17-19 rats, which is usually expressed around PN30 [50]. Specifically, Mellott et al. [24] found evidence for an enhanced activation (measured by phosphorylation) of both MAPK and CREB, in hippocampal slices of P18-P25 rats supplemented with prenatal choline, which were able to navigate using relational cues three days earlier than control rats. Thus, this capacity of choline to cause an advancement in tasks relying on the cholinergic function is compatible with our findings, and consistent with the suggestion that prenatal choline facilitates the maturation of long-term memory.

Previous studies in rodents support a well-established role of the perirhinal cortex and the parahippocampal cortex in object recognition memory [51]. Hence, a plausible explanation for the worse performance of the younger group is the delayed developmental course of these areas. Accordingly, a late development of the parahippocampal areas has been reported in relation with visual memory functions in spatial cognition [52,53]. The implication of the cholinergic system in the NOR task is supported by the findings of previous studies showing that NOR acquisition and memory performance depend on the activity of the brain cholinergic system (see [54] for a review). Consistent with this, perirhinal cortex cholinergic depletion induces deficit in NOR tasks [19] and perirhinal microinjections of the cholinergic muscarinic receptor blocker scopolamine impair visual recognition memory in monkeys [55] and rats [21]. Moreover, systemic [22] and perirhinal [23] injections of the cholinergic agonist nicotine improve performance in NOR tasks. And given that prenatal choline supplementation increases the synthesis of acetylcholine from the stores in the phospholipidic membrane components [56], this could explain the enhancement of long-term memory in the early stages of development found in this study.

Our results also suggest that this effect of choline could be temporary, which is shown by the performance of PN21-22 and PN29-31 rats in the SUP group, which matched the results observed in animals in the CTL group when they reached 30 days of age. This ‘washing out’ effect over time could negate the benefits of the dietary intervention. However, it has been found that the facilitating effect of prenatal choline on memory is evident when the demand is increased [57,58]. These results could suggest that particularly challenging tasks are required in order to see an effect of early choline supplementation in mature rats. Further studies will be necessary in order to determine the effect of prenatal choline on recognition memory in immature rats versus mature rats.

There are certain limitations in the present study. The first of these is the absence of a demonstration of intact short-term memory at 21–22 days of age in the CTL group. In this regard, it is important to point out that an unpublished study carried out in our laboratory using the same procedure revealed that rats at 21–22 days of age recognise the familiar object after a short retention interval (1 h), thus confirming previous results [38–41]. The second limitation is the use of only male rats. It is well known that sex steroid hormones are important regulators of object recognition memory in rodents (see [59] for a review). However, Cost et al. [60] observed an advantage in gonadally intact adult females in relation to male rats, but only when circulating levels of ovarian steroids, estradiol, and progesterone are elevated during the estrous cycle. Due to the fact that we used infant rats, we can dismiss the possibility that hormonal factors would have had an impact on the results reported here. Nevertheless, in future studies it will be of interest to use both female and male infant rats.

In summary, this study suggests that supplementation with choline during E12-18 modulates the development of long-term recognition memory in rats, as measured in male offspring using a novel object recognition task.

These results could be relevant for evaluating the effect of pre-natal choline supplementation on human cognitive development. A study with healthy human participants found no effect of pre-natal choline
administration on spatial-visual and episodic memory in children between 10 and 12 months of age [61]. However, a recent trial has demonstrated that infants from pregnant women supplemented with 930 mg choline/day during the third trimester of pregnancy exhibited better information-processing speeds when compared with infants from pregnant women fed with 480 mg choline/day [62]. These authors argue that, unlike the previous study their study controlled the dietary intake of choline and other nutrients. Further, Fantz, [63] showed the capacity of newborns to fixate their gaze and examine surrounding stimuli by using the recognition paradigm (see also, [64]). Likewise, several studies have shown that visual recognition of stimuli (objects and/or faces) emerges and is retained for short periods that can take seconds or minutes during the first month of life ([65,66]; see [67], for a review). In addition, evidence has been found for the existence of long-term memory (days and weeks) using novelty preference protocols in children from as young as 4–5 months of age [68], and imitation protocols (declarative memory) in infants as young as 10 months [69]. Therefore, when studying prenatal choline consumption as it relates to the development of specific cognitive abilities in human infants, it will be important to choose the correct ages as well as age-appropriate tasks. In particular, it could be of interest to apply choline supplementation to infants born preterm, where a memory deficit observed during subsequent development is associated with hippocampal damage [70–72].

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