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Prenatal choline supplementation increases sensitivity to contextual processing of temporal information

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Abstract

The effects of prenatal choline availability on contextual processing in a 30-s peak-interval (PI) procedure with gaps (1, 5, 10, and 15 s) were assessed in adult male rats. Neither supplementation nor deprivation of prenatal choline affected baseline timing performance in the PI procedure. However, prenatal choline availability significantly altered the contextual processing of gaps inserted into the to-be-timed signal (light on). Choline-supplemented rats displayed a high degree of context sensitivity as indicated by clock resetting when presented with a gap in the signal (light off). In contrast, choline-deficient rats showed no such effect and stopped their clocks during the gap. Control rats exhibited an intermediate level of contextual processing in between stop and full reset. When switched to a reversed gap condition in which rats timed the absence of the light and the presence of the light served as a gap, all groups reset their clocks following a gap. Furthermore, when filling the intertrial interval (ITI) with a distinctive stimulus (e.g., sound), both choline-supplemented and control rats rightward shifted their PI functions less on trials with gaps than choline-deficient rats, indicating greater contextual sensitivity and reduced clock resetting under these conditions. Overall, these data support the view that prenatal choline availability affects the sensitivity to the context in which gaps are inserted in the to-be-timed signal, thereby influencing whether rats run, stop, or reset their clocks.

Keywords

Interval timing; Time perception; Gaps; Attention; Time sharing; Context sensitivity; Prenatal nutrition; Neural development; Hippocampus

1. Introduction

A variety of behavioral and neurobiological evidence supports the view that adult rats given prenatal-choline supplementation have increased memory capacity and precision while being able to form more enduring memories (e.g., Cheng et al., 2008; Meck et al., 1988, 1989, 2008; Meck and Williams, 1997b, 1999; Mellott et al., 2004; Montoya et al., 2000; Williams et al., 1998 – see McCann et al., 2006 and Meck and Williams, 2003 for reviews). Recent studies have shown that prenatal choline supplementation causes long-term adaptations in

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hippocampal choline metabolism (Cermak et al., 1998, 1999) and it is well accepted that the cholinergic system has a central role in memory function (Bartus et al., 1982; Hasselmo and Giocomo, 2006). It has also been demonstrated that prenatal-choline supplementation protects against seizure-induced memory deficits (Wong-Goodrich et al., 2008; Yang, et al., 2000) while elevating the baseline level of hippocampal neurogenesis in adult rats (Aimone et al., 2006; Glenn et al., 2007). In terms of neuroplasticity, adult hippocampal slices from prenatally choline-supplemented rats exhibit a lower threshold for the induction of long-term potentiation compared with slices from control rats (Jones et al., 1999; Pyapali et al., 1998). Furthermore, perinatal choline supplementation can alter behavior and neurochemistry following a variety of developmental disorders, including the alleviation of behavioral abnormalities associated with fetal alcohol syndrome in Sprague-Dawley rats (Thomas et al., 2000, 2004, 2007; Wagner and Hunt, 2006), and the attenuation of some of the motor deficits observed in a *Mecp2^{lox}* mouse model of Rett syndrome (Nag and Berger-Sweeney, 2007). Recent reports have also demonstrated that prenatal choline supplementation improves attention and sensory gating in a DBA/2 mouse model of schizophrenia that exhibits reduced numbers of hippocampal $\alpha 7$ nicotinic receptors (Stevens et al., 2008) as well as the contextual sensitivity of Pavlovian conditioning (Lamoureux et al., 2008).

The cholinergic innervation of the hippocampus is a major target of prenatal choline manipulations. Changes in the size and shape of basal forebrain cholinergic neurons (e.g., McKeon-O'Malley et al., 2003; Williams et al., 1998) following prenatal choline manipulations are accompanied by modifications in acetylcholine turnover and choline transporter expression in the septum and hippocampus (Cermak et al., 1999; Mellott et al., 2007b), modulation of hippocampal neurogenesis, gene expression, NGF levels, and MAPK and CREB activation (e.g., Glenn et al., 2007; Mellott et al., 2004, 2007a; Sandstrom et al., 2002), changes in dendritic fields and spine density in CA1 and dentate gyrus (DG) regions of the hippocampus (Meck et al., 2008), as well as modification of the neuropathological response to status epilepticus (e.g., Holmes et al., 2002; Wong-Goodrich et al., 2008) and thresholds for eliciting long-term potentiation (LTP) in the hippocampus (Jones et al., 1999; Pyapali et al., 1998.) These findings suggest that alterations in choline availability during early development may have specific impact on the ontogeny and later functioning of basal forebrain cholinergic neurons as well as efferent neurons involved in hippocampal LTP. These findings also predict that this dietary manipulation will affect mostly behaviors that rely on the hippocampus.

Working memory for event durations is affected by lesions of the hippocampal system. For example, fimbria-fornix lesions produce a complete amnesia for the memory of the duration of the event prior to an unexpected, brief retention-interval (gap), while lesions in the frontal system had no effect on working memory for duration (Meck et al., 1984, 1987; Olton et al., 1987). Consequently, it was of interest in the present study to investigate the effects of prenatal choline supplementation on the short-term memory for event durations and the contextual processing of temporal information. These questions were addressed using the peak-interval (PI) timing procedure, in which subject's responses are reinforced for responding at a specific criterion duration. In well-trained subjects, the mean response rate increases after the onset of the to-be-timed signal, reaches a peak about the target duration and then gradually declines afterwards during unreinforced probe trials (Catania, 1970; Church, 1978; Paule et al., 1999; Penney et al. 1996). When trained in a PI procedure using both auditory (e.g., Cheng et al., 2006; Meck and Williams, 1997c) and visual signals (e.g., Cheng and Meck, 2007; Meck and Williams 1997a, c) adult rats treated prenatally with supplemental choline exhibit enhanced precision in the temporal control of responding. These beneficial effects can be related to the reduction in non-scalar sources of variance attributable to the non-temporal aspects of the procedure, as well as an increase in rats' sensitivity to the inhibitory effects of a signaled delay to reinforcement, and enhanced control over the resetting of an internal clock (e.g., Gibbon and Church, 1984; Gibbon et al., 1984; Matell and Meck 1999; Cheng et al. 2006; Cheng and

Meck, 2007). Here we address two outstanding questions relative to the role of choline supplementation in processing temporal information.

First, whether the increased memory capacity and attention gating exhibited by choline supplemented rats extends to memory for timed events, which can be tested in the PI procedure by inserting unexpected, brief breaks or gaps in the signal (Church, 1978; Roberts and Church, 1978; Fortin, 2003; Fortin and Tremblay, 2005; Fortin et al., 2008). Observed changes in the distribution of responses in trials with gaps following behavioral (Buhusi and Meck, 2000, 2006a, b) and neurobiological (e.g., Buhusi and Meck, 2002; Buhusi and Meck, 2007; Meck, 1988; Meck et al., 1984) manipulations in the PI procedure with gaps are used to address the mechanisms involved in memory for timed events. For example, when rats time a visual signal in a (standard) PI procedure, the introduction of a (dark) gap prompts rats to delay their response function by an amount approximately equal to the duration of the gap, which is taken to suggest that rodents retain in working memory the pre-gap interval and resume timing after the gap where they left off before the gap, using a stop mode (Church, 1978; Roberts and Church, 1978). However, when rats time the absence of a visual signal in a so-called reversed PI procedure (Buhusi and Meck, 2000), the introduction of a reversed, illuminated gap prompts rats to delay their response function after the gap for a duration that is approximately the sum of the gap and pre-gap intervals. This has been taken to suggest that they restart the entire timing process after the gap, using a reset mode (reviewed by Buhusi 2003; Buhusi and Meck, 2008). Such a reset mode was observed after lesions of the hippocampal system (Meck et al., 1984, 1987; Olton et al., 1987). We therefore incorporated gaps into the standard and reversed PI procedure in a manner similar to Buhusi and Meck (2000) in order to evaluate the effects prenatal choline availability on memory for timed events.

The second outstanding question is whether the increased temporal sensitivity exhibited by adult rats given prenatal choline supplementation extends to issues dealing with attention and contextual sensitivity. This issue can be addressed in the PI procedure with gaps by manipulating the relative salience of the gap to the signal (Buhusi and Meck, 2000; Buhusi et al., 2002; Buhusi et al., 2005; Buhusi et al., 2006) and the content of the signals used in the procedure (Buhusi and Meck, 2002). For example, both rodents and birds run, stop, or reset their clocks depending on gap content (Buhusi and Meck, 2002; Buhusi et al., 2002), gap discriminability (Buhusi et al., 2005, 2006), and level of visual acuity (Buhusi et al., 2005). Such results provide evidence for the hypothesis that during gaps in the to-be-timed signal resources are re-allocated (diverted away from timing) in proportion to the salience of the gap to the context in which it is presented, which includes both the to-be-timed signal and the intertrial-interval (ITI) (Buhusi 2003, Buhusi and Meck, 2008). Indeed, manipulations of the ITI, such as filling the ITI with a distinctive cue, were shown to affect the stop/reset mode in the PI procedure with gaps (Buhusi and Meck, 2002). Therefore, we also manipulated the content of the ITI in the PI procedure with gaps in a manner similar to Buhusi and Meck (2002) in order to evaluate the effects prenatal choline availability on interval timing in rats as a function of the context in which these gaps were inserted.

2. Results

2.1. Standard PI-GAP procedure

Choline-supplemented (SUP, $n=7$), choline-deficient (DEF, $n=8$), and control rats (CON, $n=7$) were trained to time the presence of a 30-s visual stimulus. All rats acquired the timing task at similar levels of performance as measured by the accuracy, precision, and peak rate of the response functions in PI trials. Three one-way ANOVAs failed to reveal differences among the treatment groups in PI trials with respect to timing accuracy (peak time of response function), $F(2,19) = 3.18$, timing precision (width of response function), $F(2,19) = 0.01$, and peak response rate (maximum height of response function), $F(2,19) = 0.35$, p 's > 0.05 . The

estimated mean peak time ($\pm$ SEM) in the standard PI trials, 30.97 ± 0.72 s, was not significantly different from the 30-s criterion, $t(21) = 1.35$, $p = 0.19$.

During testing, rats were presented with unexpected, brief (5, 10, or 15 s) dark gaps in the 30-s to-be-timed visual signal (standard PI-GAP procedure, Buhusi and Meck, 2000). The left panel of Fig. 1 shows the effect of 5-s gaps in the standard condition. The graph shows the estimated peak time in trials with gaps (y axis) and without gaps (x axis) for individual rats in each treatment group. A 5-s gap prompts the DEF rats to delay their response functions approximately 5 s (the duration of the gap) relative to PI trials, suggesting that they maintained the pre-gap time in working memory during the gap, and resumed timing after the gap (stop mode). In contrast, the SUP rats delayed their response functions approximately 20 s (the duration of the gap, 5 s, plus the duration of the pre-gap interval, 15 s), suggesting that they failed to maintain the pre-gap duration in working memory, and instead re-started timing anew after the gap (reset mode). Finally, CON rats delayed their response function in-between the stop and reset modes.

To quantify these results, a shift in peak time in gap trials relative to PI trials was computed for each rat by subtracting from the estimated peak time in gap trials, the estimated peak time in PI trials and the duration of the gap. A null shift is indicative of the stop mode, and a shift equal to the pre-gap interval (15 s) is indicative of the reset mode. The right panel of Fig. 1 shows the shift in peak time after 5, 10, or 15-s gaps in CON, DEF, and SUP treatment groups. A mixed ANOVA with factors Group and Gap duration, revealed a significant main effect of Group, $F(2,19) = 5.31$, $p < 0.05$, but no significant effect of Gap duration, $F(2,38) = 1.95$, or Group $\times$ Gap duration interaction, $F(4,38) = 1.48$, $p's > 0.05$. Post-hoc analyses indicated reliable differences between DEF and SUP rats (Scheffe test, $p < 0.02$), but not between CON and SUP, or between CON and DEF rats, $p's > 0.05$. Further analyses indicated that the shift in peak time in SUP rats is not reliably different from 15 s (i.e., they reset timing) for all gap durations, $t(6) \leq 1.25$, $p's > 0.05$, while the shift in peak time in DEF rats was significantly less than 15 s (i.e., they did not reset timing), $t(7) = 4.12$, $p < 0.01$. CON rats reliably reset for the longer 10-s and 15-s gaps, $t(6) \leq 2.48$, $p's > 0.05$, but not for the shorter 5-s gap, $t(6) = 3.60$, $p < 0.05$. Together these data indicate that rats in the SUP treatment group were more sensitive to the interruption than rats in either the CON or the DEF groups, suggesting that they displayed a high degree of context sensitivity as indicated by clock resetting after a gap.

2.2. Reversed PI-GAP procedure

The effect of a gap has been shown to be highly sensitive to the features of the context of the timing procedure (reviewed by Buhusi, 2003; Buhusi and Meck, 2008). For example, when timing the presence of a stimulus, rats tend to stop timing after a dark (standard) gap, but when timing the absence of a stimulus, they tend to reset after an illuminated gap of the same duration (Buhusi and Meck, 2000). Therefore, we evaluated the sensitivity of the rats in the CON, DEF, and SUP treatment groups to non-temporal features of the gap procedure, by reversing the content of the stimuli while maintaining the temporal parameters of the protocol.

Rats were trained to time the absence of a 30-s visual stimulus in a reversed PI-GAP procedure (Buhusi and Meck, 2000), and then tested with brief (1, 5, and 10-s) illuminated gaps. Three one-way ANOVAs failed to reveal differences among the CON, DEF, and SUP treatment groups in reversed PI trials with respect to timing accuracy, $F(2,19) = 0.14$, timing precision, $F(2,19) = 0.93$, and peak response rate, $F(2,19) = 0.94$, $p's > 0.05$. The estimated mean peak time ($\pm$ SEM) in the standard PI trials, 32.41 ± 1.35 s, was not significantly different from the 30-s criterion, $t(21) = 1.79$, $p > 0.05$, suggesting that rats in all treatment groups acquired the timing task at similar levels of performance.

When the rats were tested with reversed 1, 5, and 10-s gaps all treatment groups reset their timing as illustrated in Fig. 2. Indeed, a mixed ANOVA with factors Group and Gap duration failed to reveal any main effects of Group, $F(2,19) = 0.36$, and Gap duration, $F(2,38) = 0.03$, or Group $\times$ Gap duration interaction, $F(4,38) = 0.35$, p 's > 0.05 . The shift in peak time in gap trials relative to PI trials was not reliably different from 15 s (reset mode), for either the CON, $t(6) = 2.37$, DEF, $t(7) = 0.67$, or SUP, $t(6) = 0.4$ groups, p 's > 0.05 . Together these data indicate that all treatment groups were sensitive to reversing the content of the timed signals and gaps.

2.3. Standard PI-GAP procedure with filled intertrial intervals

While reversing the signals prompts rats to switch their response mode from stop to reset after a gap in the visual signal (Buhusi and Meck, 2000), a switch in mode from reset to stop was observed in rats when the inter-trial interval (ITI) was filled with an auditory stimulus (Buhusi and Meck, 2002), suggesting that rats are highly sensitive to contextual manipulations of the PI-GAP procedure. Therefore, we tested the sensitivity of the rats in the CON, DEF, and SUP treatment groups to contextual manipulations features of the standard PI-GAP procedure by filling the ITI with white noise.

Rats were trained to time the presence of a 30-s visual stimulus in a standard PI-GAP procedure in which the ITI was dark and noisy (Buhusi and Meck, 2000), and were subsequently tested with brief (5, 10, and 15-s) dark, silent gaps. Three one-way ANOVAs failed to reveal differences among the CON, DEF, or SUP treatment groups in reversed PI trials in respect to timing accuracy, $F(2,19) = 0.87$, timing precision, $F(2,19) = 1.01$, and peak response rate, $F(2,19) = 0.95$, p 's > 0.05 . This finding suggests that rats in all treatment groups acquired the timing task at similar levels of performance. The estimated mean peak time ($\pm$ SEM) in the standard PI trials, 32.99 ± 1.03 s, was significantly larger than the 30-s criterion, $t(21) = 2.90$, $p < 0.05$.

The effect of filling the ITI when testing with standard 5, 10, and 15-s gaps is shown in Fig. 3. A mixed ANOVA with factors Group and Gap duration failed to reveal any main effects of Group, $F(2,19) = 0.63$, and Gap duration, $F(2,38) = 0.72$, or the Group $\times$ Gap duration interaction, $F(4,38) = 0.18$. p 's > 0.05 . Further analyses indicated that in the CON and SUP treatment groups the shift in peak time in gap trials relative to PI trials was reliably smaller than 15 s (reset mode) for rats in the CON and SUP treatment groups, but not for rats in the DEF group, which reliably reset after 10-s and 15-s gaps, but not after a 5-s gap. Together these data indicate that rats in the SUP group are as sensitive as CON rats to the stopping effect of filling the ITI, but that rats in the DEF group are significantly less sensitive to this manipulation.

2.4. Summary of PI-GAP results

The overall PI-GAP results as a function of treatment group, condition (standard, reversed, and filled ITI) and gap duration (1, 5, 10, and 15 s) are illustrated in Fig. 4. The graph indicates that relative to the standard condition, rats in both the CON and SUP treatment groups reset their timing in the reversed condition, but stop their clocks in the filled-ITI condition. In contrast, rats in the DEF treatment group reset their timing in the reversed condition, but are less sensitive to contextual effects in the filled-ITI condition. This latter suggestion was further supported by a joint analysis of data in the standard and filled-ITI conditions. A mixed ANOVA with factors Group, Gap duration, and Condition reveal a reliable main effect of condition (standard vs. filled ITI), $F(1,19) = 5.22$, and Group $\times$ Condition interaction, $F(2,19) = 4.11$ (p 's < 0.05), but no other significant main effects or interactions. Planned comparisons indicated a significant stopping effect of filling the ITI in the CON, $F(1,19) = 4.42$ and SUP rats, $F(1,19) = 7.28$ (p 's < 0.05), but not in the DEF rats, $F(1,19) = 0.98$, $p > 0.05$. Together these data indicate that rats in the CON and SUP treatment groups, but not rats in the DEF group, use contextual information about the ITI to flexibly modulate their behavior in the PI-GAP

procedure. In conjunction with the finding that SUP rats are more sensitive to the gap in the standard PI condition, as demonstrated by their propensity to reset timing, these results suggest that prenatal choline availability affects the sensitivity to the context in which gaps are inserted in the to-be-timed signal, thereby influencing whether rats run, stop, or reset their clocks.

3. Discussion

Previous research indicates that the effect of a gap in a to-be-timed signal is highly sensitive to the features of the context of the procedure (reviewed by Buhusi, 2003; Buhusi and Meck, 2008). For example, increasing the salience (Buhusi and Meck, 2000; Buhusi et al., 2005; Buhusi et al., 2006) of the to-be-timed signal prompts rats to switch from stopping their clocks to resetting their clocks after a gap in the signal. On the other hand, a switch from the reset mode to the stop mode was observed when increasing the duration of the ITI (Buhusi and Meck, 2008) or filling the ITI with a highly distinctive signal (Buhusi and Meck, 2002.) Therefore, we evaluated memory for time and contextual sensitivity as a function of prenatal choline availability using the PI procedure with gaps by (a) manipulating the duration of the gap (standard condition), (b) reversing the content of the stimuli (reversed condition), and (c) filling the ITI with an auditory stimulus (filled ITI condition), while maintaining all other temporal parameters of the procedure. We found that rats in the SUP treatment group were more sensitive to the gap than rats in the CON and DEF groups, in both resetting in the standard condition and stopping when filling the ITI, thus demonstrating a superior control over the resetting of an internal clock. In contrast, DEF rats showed reliably less sensitivity to contextual manipulations; they did not reset in the standard condition, and were not able to further stop their clocks in the filled ITI condition, thus demonstrating limited attentional control over the starting and resetting of an internal clock (Buhusi and Meck, 2008; Cheng et al., 2006; Meck, 1984).

Previous work has also shown that both the frontal and temporal systems are involved in memory for timed events, but in complementary ways. A double dissociation resulted from lesions of the frontal cortex and the nucleus basalis magnocellularis, on the one hand, and the hippocampus, fimbria-fornix, and the medial septal area, on the other. Lesions in the frontal system produce a rightward shift (overestimation) of the expected time of reinforcement, while lesions in the hippocampal system produce a leftward shift (for review see Buhusi and Meck, 2005; Gibbon et al., 1997; Meck, 1996, 2002; Meck et al., 1986, 1987). Dissociation was also found in the effects of these lesions on working memory for the duration of a stimulus prior to a gap or retention interval interpolated during that stimulus. Fimbria-fornix lesions produce a complete amnesia for the memory of the duration of the event prior to the gap, while lesions in the frontal system had no effect (Meck et al., 1984, 1987; Olton et al., 1987). In turn, modifications in acetylcholine turnover and choline transporter expression in the septum and hippocampus (Cermak et al., 1999; Mellott et al., 2007b), modulation of hippocampal neurogenesis, gene expression, NGF levels, and MAPK and CREB activation (e.g., Glenn et al., 2007; Mellott et al., 2004, 2007a; Sandstrom et al., 2002), changes in dendritic fields and spine density in CA1 and dentate gyrus (DG) regions of the hippocampus (Meck et al., 2008), as well as modification of the neuropathological response to status epilepticus (e.g., Holmes et al., 2002; Wong-Goodrich et al., 2008) and thresholds for eliciting long-term potentiation (LTP) in the hippocampus (Jones et al., 1999; Pyapali et al., 1998) accompany changes in the size and shape of basal forebrain cholinergic neurons (e.g., McKeon-O'Malley et al., 2003; Williams et al., 1998) following prenatal choline manipulations. These findings suggest that alterations in choline availability during early development may have specific impact on the ontogeny and later functioning of basal forebrain cholinergic neurons as well as efferent neurons involved in hippocampal LTP. Together, these results suggest that prenatal choline availability affects contextual processing of temporal information by means of changes in hippocampal function (Lamoureux et al., 2008), or hippocampal interaction with the other

brain areas involved in time perception, notably cortico-striatal circuits (Buhusi and Meck, 2005; Cheng et al., 2007a, b; Meck, 1988, 2006a, b, c, 2007).

A description of the neurobiological mechanisms involved in interval timing is provided by the striatal beat-frequency (SBF) model, which ascribes a role for detecting event durations to medium spiny neurons within the dorsal striatum (e.g., Buhusi and Meck, 2005; Lustig et al., 2005; MacDonald and Meck, 2004; Matell and Meck, 2000, 2004). These striatal neurons have a set of functional properties that place them in an ideal position to detect behaviorally relevant patterns of afferent cortical input and reflect alteration in clock speed (e.g., Beiser and Houk, 1998; Lustig and Meck, 2005; Matell et al., 2003, 2004, 2006). Briefly, the SBF model posits that medium spiny neurons in the dorsal striatum become entrained to fire in response to oscillating, coincident cortical inputs that become active at previously trained event durations. This timing model is particularly useful insofar as the striatal neurons modeled using the SBF framework behave as they do when assessed using multiunit electrical recordings during interval-timing procedures (Matell et al., 2003 – see also Hinton and Meck, 2004). However, the exact nature of the horizontal shifts in timing functions as a result of attentional and/or clock speed manipulations and prenatal choline availability is as yet unresolved within the context of the SBF model (see Cheng et al., 2006). One proposal is that tonic dopamine levels within the striatum modulate the oscillatory frequency within the cortex through cortico-striato-thalamo-cortical feedback mechanisms (e.g., Bamford et al., 2004a, b; Buhusi and Meck, 2005; Lustig et al., 2005; Matell and Meck, 2004; Meck, 2006a, b). The observation that timing and attention to time are sensitive to prenatal choline availability further supports the view that differential activation of cortical, hippocampal, and striatal systems is critical for contextual processing and the control of the resetting of an internal clock (e.g., Buhusi and Meck, 2005; Cheng et al., 2006; Lamoureux et al., 2008; Lustig and Meck, 2001; Matell and Meck, 1999; Meck, 2005; Meck and Benson, 2002; Meck and Williams, 2003).

4. Experimental procedures

4.1. Animals and housing

Subjects—Twenty-two naive male Sprague-Dawley CD strain rats, the offspring of timed-pregnant dams obtained from Charles River Laboratories (Kingston, NY), served as subjects. All dams were obtained on embryonic day (ED) 9 and fed AIN-76A purified synthetic diet containing 1.1 g/kg choline chloride (Dyets, Bethlehem, PA) and water *ad libitum*. The manipulation of dietary choline took place from the evening of ED 11 through the morning of ED 18, after which all dams were fed standard purified diet. During the treatment period, all dams received drinking water containing 50 mM saccharin. Choline was supplemented (SUP) for one group of dams (n=4) with the addition of 25 mM choline chloride in the saccharin drinking water. A second group of dams (n=4) received drinking water without choline and was switched to a purified diet completely deficient (DEF) of choline. The third group of dams (n=4) served as a control (CON) and was maintained on the standard purified diet (1.1 g/kg choline chloride) and drinking water without added choline. Dams were housed individually in clear polycarbonate cages (27.9 × 27.9 × 17.8 cm) in a colony room maintained on a 12–12 h, light-dark cycle.

Litters of 10 male pups, each containing rats from all dietary choline treatments (SUP, DEF, and CON), were cross-fostered at birth to untreated control dams. Pups were weaned at 24 days postnatal (PD 24), housed five/cage at PD 30, and then two/cage on PD 45. These offspring received the standard AIN-76A diet described above and tap water *ad libitum* from weaning until PD 90. Throughout the remainder of the study, the rats were maintained at 85% of their *ad libitum* weights by limiting their access to food; water was still available continuously in the home cages. Twenty-two male rats: DEF = 8, CON = 7, and SUP = 7 were selected for study. Experiments were conducted during the light portion of the L/D cycle in accordance

with standard procedures approved by the Institutional Animal Care and Use Committee of Duke University.

4.2. Apparatus

All experimental data were obtained in identical lever boxes (MED Associates, Model ENV-007) housed in light and sound attenuating cubicles (MED Associates, Model ENV-019). Each lever box had inside dimensions of approximately 24 cm × 31 cm × 31 cm. The top, sidewalls, and door were constructed of clear acrylic plastic. The front and back walls were constructed of aluminum, and the floor was comprised of 19 parallel stainless steel bars. Each lever box was equipped with two retractable response levers (MED Associates, Model ENV-112) situated on the front wall of the lever box. Precision food pellets (45 mg; Research Diets, New Brunswick, NJ) could be delivered by a pellet dispenser (MED Associates, Model ENV-203) to a food cup on the front wall 1 cm above the floor. A 28-V, 170-mA, 2,500-lx house-light was mounted at the center-top of the front wall. A white noise amplifier/speaker system (MED Associates, Model ENV-225) was mounted on the opposite wall from the levers. Each lever box was housed inside a sound and light attenuated chamber that was equipped with an eyepiece viewer for observation and a ventilation fan that produced a 60-dB background noise. An IBM-PC compatible computer attached to an electronic interface (MED Associates, Model DIG-700 and SG-215) was used to control the experimental equipment and record the data.

4.3. Magazine and lever training

Each rat received 5 sessions of combined magazine and lever training during which a food pellet was delivered once each minute for 60 min (magazine training), and in addition, each lever press produced food. The left retractable lever was extended into the chamber and 10 lever presses were reinforced; then the left lever was retracted and the right retractable lever was extended into the chamber and 10 lever presses were reinforced; then the right lever was retracted and the left lever was again extended. This alternation between levers continued until the rat had pressed each lever 30 times or 60 min had passed, whichever came first. The house light illuminated the chamber during these sessions.

4.4. Standard fixed-interval training (Sessions 1–10)

For each daily session all rats received 64 fixed-interval (FI) trials during which the house light was turned on for the to-be-timed interval; the first lever press 30 s after the onset of the visual signal was reinforced by the delivery of a food pellet and turned off the house light for the duration of the random intertrial interval (ITI). Trials were separated by a 60 ± 30 s random ITI, which was silent and dark.

4.5. Standard peak-interval testing with gaps (Sessions 11–31)

During each session the rats received 32 FI trials randomly intermixed with 32 non-reinforced peak-interval (PI) trials in which the visual signal was presented for a duration three times longer than the FI, before being terminated irrespective of responding. Trials were separated by a 60 ± 30 s random ITI (silent, dark). During the last two (test) sessions, rats received 32 FI trials randomly intermixed with 32 non-reinforced test trials (8 trials for each of the four types of test conditions). Eight test trials were standard PI trials during which the visual to-be-timed stimulus was not interrupted. During the remaining 24 test trials, half-way into the to-be-timed interval, i.e., 15 s after the beginning of the visual signal, the timed visual signal was interrupted by either a 5-s, 10-s, or 15-s dark gap (8 test trials each). At the offset of the gap, the to-be-timed visual signal was reinstated for a duration matching that of the PI trials, and then terminated independently of responding for the 60 ± 30 s random duration of the ITI.

(silent, dark). The sum of the pre-gap, gap, and post-gap durations was equal to the duration of the to-be-timed visual signal in PI trials.

4.6. Reversed peak-interval testing with gaps (Sessions 32–56)

These sessions were identical in terms of temporal parameters with those described above except the stimuli were reversed: the to-be-timed signal was 30-s of the absence of the visual signal (dark) and the ITI was illuminated. During the last three (test) sessions, rats received 32 reversed FI trials randomly intermixed with 32 non-reinforced test trials (8 trials for each of the four types of test conditions). Eight test trials were reversed PI trials during which the visual to-be-timed stimulus (dark) was not interrupted. During the remaining 24 test trials, half-way into the to-be-timed interval, i.e., 15 s after the beginning of the visual signal, the timed visual signal was interrupted by either a 1-s, 5-s, or 10-s illuminated gap (8 test trials each). At the offset of the gap, the to-be-timed visual signal was reinstated for a duration matching that of the PI trials, and then terminated independently of responding for the 60 ± 30 s random duration of the ITI (silent, illuminated). The sum of the pre-gap, gap, and post-gap durations was equal to the duration of the to-be-timed visual signal in PI trials.

4.7. Standard peak-interval testing with filled intertrial intervals (Sessions 57–80)

These sessions were otherwise identical with the standard PI testing except an auditory stimulus (white noise) was presented throughout the inter-trial interval. During the last three (gap) sessions, rats received 32 FI trials randomly intermixed with 32 non-reinforced test trials (8 PI trials, and 8 dark gap trials as described above). Trials were separated by a 60 ± 30 s random ITI, which was dark and noisy. Please see Table 1 for an outline of the experimental conditions.

4.8. Data analysis

The experimental procedures were controlled through a MED Associates interface connected to an IBM-PC compatible computer running a MED-PC software system (MED Associates, 1999). Lever presses were recorded in real time. Lever presses recorded during test (gap) trials were used to estimate the peak time, peak rate, and precision of timing (width of the response functions) for each rat. The number of responses (in 5-s bins) was averaged daily over trials, to obtain a mean response rate function for each rat. Analyses were conducted on the data from an interval twice as large as the FI (i.e., 60 s), starting at the onset of the to-be-timed signal (for data in standard PI trials) or at the offset of the gap/distracter (for gap, distracter, and gap +distracter trials). The mean absolute response rate in the interval of interest was fit using the Marquardt-Levenberg iterative algorithm (Marquardt, 1963) to find the coefficients (parameters) of a Gaussian+linear equation that gave the “best fit” (least squares minimization) between the equation and the data (Buhusi and Meck, 2000). This algorithm provides the following parameters of the response curve: the accuracy of timing (response peak time, parameter t_0), precision of timing (width of response function, parameter b), and peak rate of response (parameter $a+d$, for details see Buhusi and Meck, 2000). A shift in gap trials relative to PI trials was computed by subtracting from the estimated peak time t_0 in gap trials the estimated peak time t_0 in PI trials and the duration of the gap. These parameters were submitted to statistical analyses. All statistical tests were evaluated at a significance level of 0.05.

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Fig. 1. Standard peak-interval testing with gaps

The effect of standard (dark) gaps in a to-be-timed visual signal in choline-supplemented (SUP), choline-deficient (DEF), and control rats (CON). **A**: Response functions in unreinforced probe trials and 5-s gap trials. **B**: Individual rat data. **C**: Group mean ($\pm$ SEM) data.

Fig. 2. Reversed peak-interval testing with gaps

Mean ($\pm$ SEM) shifts in peak time as a function of the reversed (iluminated) gaps in a to-be-timed dark visual signal in choline-supplemented (SUP), choline-deficient (DEF), and control rats (CON).

Fig. 3. Peak-interval testing with gaps with filled intertrial intervals (ITI)
Mean ($\pm$ SEM) shifts in peak time as a function of the effect of filling the dark ITI with an auditory stimulus in choline-supplemented (SUP), choline-deficient (DEF), and control rats (CON).

Fig. 4. Summary of the standard gap, reversed gap, and filled intertrial interval (ITI) conditions
Mean ($\pm$ SEM) shifts in peak time in gap trials relative to trials without gaps in choline-supplemented (SUP), choline-deficient (DEF), and control rats (CON) are plotted as a function of standard, reversed, and filled-ITI conditions.

TABLE 1**EXPERIMENTAL CONDITIONS**

Condition	Standard	Reversed	Filled ITI
To-be-timed stimulus	light	dark	light
Intertrial interval	dark	light	dark and noisy
Gap stimulus	dark	light	dark
Criterion duration	30s	30s	30s
Gap duration	5 s, 10 s,15 s	1 s, 5 s,10 s	5 s,10 s,15 s