Published in final edited form as:

Brain Res. 2012 March 14; 1443: 52–63. doi:10.1016/j.brainres.2012.01.018.

Supplemental dietary choline during development exerts antidepressant-like effects in adult female rats

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Abstract

Perinatal choline supplementation in rats is neuroprotective against insults such as fetal alcohol exposure, seizures, and advanced age. In the present study we explored whether dietary choline supplementation may also confer protection from psychological challenges, like stress, and act as a natural buffer against stress-linked psychological disorders, like depression. We previously found that choline supplementation increased adult hippocampal neurogenesis, a function compromised by stress, lowered in depression, and boosted by antidepressants; and increased levels of growth factors linked to depression, like brain-derived neurotrophic factor. Together, these were compelling reasons to study the role of choline in depressed mood. To do this, we treated rats with a choline supplemented diet (5 mg/kg choline chloride in AIN76A) prenatally on embryonic days 10–22, on postnatal days (PD) 25–50, or as adults from PD75 onward. Outside of these treatment periods rats were fed a standard diet (1.1 mg/kg choline chloride in AIN76A); control rats consumed only this diet throughout the study. Starting on PD100 rats' anxiety-like responses to an open field, learning in a water maze, and reactivity to forced swimming were assessed. Rats given choline supplementation during pre- or post-natal development, but not adult-treated rats, were less anxious in the open field and less immobile in the forced swim test than control rats. These effects were not mediated by a learning deficit as all groups performed comparably and well in the water maze. Thus, we offer compelling support for the hypothesis that supplemental dietary choline, at least when given during development, may inoculate an individual against stress and major psychological disorders, like depression.

Keywords

nutrition; depression; hippocampus; adult neurogenesis; anxiety; behavioral despair

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Final Disclosures

None.

1. Introduction

Disorders of mental health are notably abundant in the U.S. with over a quarter of the adult population living with one of several diagnosable conditions. Accounting for nearly half of those numbers are disorders of mood, including depression, anxiety, and schizophrenia (NIMH, 2008). Furthermore, the incidence of certain disorders, depression in particular, is, on average, twice as common among females than males. Given these alarming statistics, research aimed at novel explanations and therapeutic strategies is clearly warranted. In the present study we sought evidence that choline nutriture during development and in adulthood may alter female rats' behavioral responses in the forced swimming paradigm, which is designed to reproduce the core depressive symptom of behavioral despair.

According to the Diagnostic and Statistical Manual of Mental Disorders-IV (1994), major depressive disorder is characterized by a lowered mood state, feelings of worthlessness or guilt, social withdrawal and agitation, cognitive deficits, and somatic symptoms. Individuals diagnosed with depression often report a loss of pleasure in activities they used to enjoy, hopelessness, and despair. In addition, a substantial portion of depressed individuals also report anxiety (e.g. Zender & Olshansky, 2009) and when depression and anxiety are comorbid there is often a resistance to treatment with symptoms taking longer to resolve in response to antidepressants and therapy (Petersen et al., 2009; also see Lee & Dunner, 2008). In terms of etiology, there is a constellation of genetic, biological, and environmental factors that contributes to the onset and progression of depression (reviewed and discussed by Brown, Varghese, & McEwen, 2005; McEwen, 2005; 2008; Swaab, Bao, & Lucassen, 2005; also see Caspi et al., 2003). Thus, factors which may mitigate the harmful effects of these contributing factors on an organism may reduce their risk for and/or improve their prognosis with a mood disorder. In this report we present compelling evidence that the nutrient choline may be a factor that does this.

The idea of using nutrients to treat or protect individuals against disorders of mental health is relatively new despite an abundant literature that clearly demonstrates the impact dietary factors have on brain, body, and behavior (reviewed by Gómez-Pinilla, 2008; Williams, 2008; also see Rogers, 2001). Choline is no exception: classified as a vital nutrient, it serves many and varied functions in the body and brain (Blusztajn, 1998; Zeisel, 2006; Zeisel & da Costa, 2009). Mammals must consume the majority of the choline required for normal bodily functions in their diet; choline is found in various concentrations in many foods, but is particularly plentiful in legumes, grains, meats, vegetables, and eggs (Zeisel & da Costa, 2009). Fortunately, the body is able to produce choline *de novo* in the liver and may therefore buffer the organism over periods of lowered choline intake. This may also be taken as an indication of the vital processes subserved by choline. For examples, it is essential for the construction and integrity of cell membranes, participates in a number of cell signaling pathways, affects gene expression by altering DNA methylation through methyl group donation, and it is the precursor to the neurotransmitter acetylcholine (Sanders & Zeisel, 2007; Zeisel & da Costa, 2009).

Two decades of research have firmly established that prenatal choline supplementation improves attention and memory and protects against age-related declines in cognitive function in both male and female rats (Meck & Williams, 1997b, 2003; 2007). These remarkable behavioral findings suggest that dietary supplementation of choline during development has life-long, positive consequences for neural function. Fitting extraordinarily well with these behavioral results are physiological and neural findings that the basal forebrain-hippocampal cholinergic system's function and morphology are altered by the levels of choline *in utero* (Albright et al., 1999; Blusztajn et al., 1998; Craciunescu et al., 2003; Pyapali et al., 1998; Williams et al., 1998; also see Loy et al., 1991). Extending this

work are findings that prenatal choline supplementation is neuroprotective against a variety of neural insults (Guo-Ross et al., 2003; Holmes et al., 2001; Thomas et al., 2000; 2007; Wong-Goodrich et al., 2008a; Yang et al., 2000) and that prenatal choline deficiency may reduce the capacity for neuroplastic changes in adult brains (Glenn et al., 2007).

Consistent with a neuroprotective role for choline, we recently reported (Glenn et al., 2007; also see Glenn et al., 2008) that prenatal choline supplementation markedly increased adult hippocampal neurogenesis and levels of the growth factor, brain-derived neurotrophic factor (BDNF). These findings are remarkably consistent with others offered in support of a hypotheses that a failure in neuroplasticity is a central component in depression (Angelucci et al., 2005; Eisch et al., 2008; Jacobs, van Praag, Gage, 2000; Kempermann & Kronenberg, 2003; Malberg, 2004; Malberg & Schechter, 2005; Paizanis, Hamon, Lanfumey, 2007; Vollmayr, Mahlstedt, Henn, 2007). Specifically, depression is associated with a failure in adult hippocampal neurogenesis and low levels of BDNF (Castrén, Vöikar, Rantamäki, 2007; Duman & Monteggia, 2006; Sapolsky, 2004; Schmidt & Duman, 2007; Shimizu et al., 2003) and antidepressants have in common the ability to increase both (Castrén, 2004; Dranovsky & Hen, 2006; Martinowich, Manji, & Lu, 2007; Santarelli et al., 2003; Shimizu et al., 2003). That adult rats treated with prenatal choline supplementation are, in essence, displaying upregulation in processes that normally require drug treatment is compelling evidence in support of a hypothesis that choline-supplemented rats may be more resilient in the face of stress and not as likely to display behavioral patterns that are consistent with lowered mood state. Thus, given the common mechanism of action of choline and antidepressant drugs on the brain, the present study was designed to uncover whether choline could exert an antidepressant-like effect in behavior.

To test this hypothesis, we administered a choline-supplemented or standard diet to female rats at different points in their lifespan. Female rats were selected based on the preponderance of depression in human females and a paucity of research on females in the animal literature. We used the forced swimming test, which is widely used and documented as an efficacious indicator of the success of novel pharmaceutical therapies for depression (reviewed in Cryan et al., 2005). We also assessed spatial learning in the water maze as a means of insuring that our forced swimming results were unlikely to be accounted for by reactivity to the water and swimming or learning abilities. As an additional index of emotionality, we used the open field test to evaluate anxious-like behavior and activity levels in the rats as a function of the diet treatments. Given the propensity for there to be elevated anxiety in depression (Zender & Olshansky, 2009), and for antidepressants to also be anxiolytic (Bespalov, van Gaalen, & Gross, 2010), discovering the extent to which choline levels may affect this behavior would provide added support to the hypothesis that choline has antidepressant properties. Overall, the results of the present research will provide support for the provocative idea that dietary factors are useful targets in the management and treatment of depression and other mood disorders. In particular, this work offers an exciting and relatively simple strategy for supplementing modern pharmaceutical and other therapeutic approaches.

2. Results

2.1 Rats and Choline Diets

During the course of all diet manipulations, we regularly measured food and water intake and body weight. We detected no statistically significant differences in these measures at any time over the course of the study (data not shown). The experimental design and timeline of the research is displayed in Fig. 1.

2.2 Open Field: Activity and Anxiety

Fig. 2 shows measures of activity and anxiety-like behavior (overall and center exploration) that were collected during the 5-minute open field test. A one-way between subjects ANOVA comparing the 4 diet groups on the percentage of time spent moving in the field was statistically significant ($F[3,39]=3.412$, $p=0.028$; see Fig. 2A), however post-hoc Dunnett tests revealed that none of the choline-supplemented groups were significantly different from the CONTROL group. As can be seen in Fig. 2A, the overall effect of diet was due to lower overall movement in the ADULT-SUP rats, particularly in comparison to the PRE-SUP ($p=0.005$) and, to a lesser extent, POST-SUP rats ($p=0.08$).

An ANOVA comparing the 4 groups on the percentage of the field explored was also statistically significant ($F[3,39]=4.686$, $p=0.007$; see Fig. 2B). In this case Dunnett tests revealed that the PRE-SUP and POST-SUP rats explored the field significantly more than the CONTROL rats ($p=0.008$ and $p=0.001$, respectively), whereas CONTROL rats were not significantly different from ADULT-SUP rats ($p=0.333$). A similar pattern was evident for the percentage of time rats spent in the center of the field, however the ANOVA was not statistically significant ($F[3,39]=2.338$, $p=0.091$; see Fig. 2C). Planned comparisons revealed that, like with the exploration measure, PRE-SUP and POST-SUP rats spent significantly more time in the center of the open field than CONTROL rats ($p=0.04$ and $p=0.008$, respectively). In addition there was also a tendency, though not significant, for ADULT-SUP rats to also spend more time in the center than CONTROL rats ($p=0.063$).

2.2 Water maze: Spatial Learning and Memory

Fig. 3 shows the performance of rats during the training and probe trials in the water maze. The latencies of rats to locate the fixed, hidden platform in the water maze on the 4 trials on each of the 3 days of acquisition were averaged and a 4×3 mixed factorial ANOVA was conducted to examine the effects of the between-subjects Diet variable and the within-subjects variable of Day of training. The main effect of Day was statistically significant ($F[2,72]=25.47$, $p=0.001$), arising from clear decreases in rats' latencies to find the hidden platform over the course of training (see Fig. 3A). However, the main effect of Diet and the interaction between Day and Diet were not statistically significant; all groups of rats learned the task at a similar rate.

One-way ANOVAs were used to analyze probe trials, in which the platform was removed from the pool; the dependent measure was the percentage of time rats spent in the quadrant that previously contained the platform on training trials. The first probe trial was conducted on the last day of acquisition and the second probe trial was conducted a week later. The results of the two ANOVAs were not statistically significant. Furthermore, one-sample t-tests comparing each groups' performance on the first probe trial to 25%, the value predicted by random searching, were all statistically significant ($p's < 0.03$; See Fig. 3B). On the second probe trial, there was a non-significant tendency for the ADULT-SUP rats to spend less time in the target quadrant than CONTROL rats and one-sample t-tests revealed that all groups ($p's < 0.03$) except the ADULT-SUP group ($p=0.193$) spent significantly more time in the target quadrant (see Fig. 3C).

A 4×4 mixed factorial ANOVA with Diet and Trial as factors was conducted on the escape latencies of rats during reversal training on the day following the second probe test and revealed a significant main effect of Trial ($F[3,96]=7.657$, $p=0.001$), indicating that rats learned the platform's new location during the session (data not shown). It was also the case that all groups learned at similar rates: neither the main effect of Diet nor the interaction between Diet and Trial were statistically significant.

2.3 Forced Swim Test: Behavioral Despair

Dependent measures collected during swim sessions, specifically latency to the first record of immobility (no movement for at least 3 seconds) and total time spent immobile, were assessed using one-way ANOVAs; mixed factorial ANOVAs with diet and minute as factors were conducted on times spent immobile during each minute of each session: 4×10 for the first, 10-minute 'induction' session (see Fig. 4) and 4×5 for the second, 5-minute 'assessment' session (see Fig. 5). There were no statistically significant differences between the groups in their latencies to become immobile for the first time (see Fig. 4A), or in the total amount of time spent immobile during the 10-minute test (see Fig. 4B). There was a significant main effect of Minute of the test on time spent immobile ($F[9,279]=80.132$, $p=0.001$), with all groups showing increased immobility over time (see Fig. 4C). Though there was neither a main effect of Diet nor a Diet × Minute interaction, there was a clear tendency for the choline-supplemented rats, particularly the PRE-SUP rats, to display less immobility overall (see Fig. 4B) and on minutes 4, 5, and 10 (see Fig. 4C).

An ANOVA conducted on the latency to immobility during the second swim session was not statistically significant ($F[3,39]=1.808$, $p=0.163$), however planned comparisons revealed that PRE-SUP and POST-SUP rats took significantly longer to become immobile than CONTROL rats ($p=0.048$ and 0.011 , respectively; see Fig. 5A). A similar trend was seen when comparing ADULT-SUP and CONTROL rats ($p=0.058$). Consistent with these results, an ANOVA conducted on the total amount of time spent immobile was statistically significant ($F[3,39]=5.363$, $p=0.004$; see Fig. 5B). Dunnett tests revealed that the PRE-SUP rats spent significantly less time immobile than CONTROL rats ($p=0.001$), whereas POST-SUP and ADULT-SUP did not ($p=0.121$ and $p=0.482$, respectively). The analysis over each minute of the second swim session revealed a significant main effect of Minute ($F[4,144]=38.328$, $p=0.001$), with all groups displaying more immobility over the course of the test (see Fig. 5C). The main effect of Diet and the interaction between Diet and Minute were also statistically significant ($F[3,36]=5.353$, $p=0.004$ and $F[12,144]=2.074$, $p=0.022$, respectively). Follow-up tests revealed statistically significant differences between the PRE-SUP rats and CONTROL rats in duration of immobility on minutes 1 ($p=0.001$), 2 ($p=0.001$), 3 ($p=0.003$), and 4 ($p=0.039$) and between the POST-SUP and CONTROL rats on minutes 1 ($p=0.005$), 2 ($p=0.044$), and 3 ($p=0.023$). The ADULT-SUP and CONTROL rats did not significantly differ on any minute of the test.

3. Discussion

The primary objective of the present study was to assess the extent to which supplemental levels of the nutrient choline, administered at different stages of development or in adulthood, may exert antidepressant effects in a behavioral model of depressive-like symptomology. Pursuant to the hypothesis that the enhancement of adult cognition and the prevention of cognitive decline with aging in rats treated with prenatal choline supplementation arises from increased neural plasticity, particularly in the hippocampus, we previously reported a marked increase in adult hippocampal neurogenesis in the dentate gyrus that was accompanied by significant increases in hippocampal levels of the growth factor, BDNF (Glenn et al., 2007). Given the importance of both adult neurogenesis and BDNF to the efficacy of antidepressants and their established role in the tale of depression (reviewed in Duman & Monteggia, 2006 and Samuels & Hen, 2011), it was a timely and novel endeavor to seek evidence that choline may have behavioral effects that resemble those of antidepressants. The results from the present study clearly and compellingly support this hypothesis. Of particular note are the findings that choline supplementation given to developing rats, either pre- or postnatally, led to an antidepressant effect in the forced swim test and less anxiety in the open field. Overall, this was not evident in rats given choline supplementation in adulthood, even when they were still consuming the choline

supplemented diet. Thus, it is likely that supplemental choline is exerting an impact on neural systems during development that persists into adulthood and renders rats more resilient in the despair-inducing procedures used.

3.1 Prenatal and postnatal, but not adult, choline supplementation reduces immobility in response to forced swimming

The most marked behavioral effects in the present study were observed in the forced swim test of behavioral despair. Rats that were choline supplemented *in utero* displayed the least amount of immobility in the forced swim tests when assessed as adults. These results are notably consistent in magnitude to others reported in the field following antidepressant treatment (e.g. see Porsolt et al., 1978). Thus, the actions of supplemental dietary choline during prenatal brain development results in an intrinsic antidepressant potential that emerges in the face of despair-inducing events that are normally attenuated by active antidepressant drugs. The lower levels of immobility in the forced swim tests could reflect overall reductions in general activity levels. However, we were able to gauge activity levels in the open field and did not observe any difference in how much they moved in comparison to the control rats (also see Glenn et al., 2007; 2008). We also did not observe any evidence that they were particularly reactive to the swimming experience in the water maze component of the study. Thus, a viable interpretation of the data from the forced swim sessions is that prenatal choline supplementation led that group of adult rats to have a qualitatively different experience in the first session that resulted in the larger differences that were observed in the second session. Supporting this view are the findings that the postnatal choline supplemented rats were not different from controls in the amount of immobility displayed during the first swim session but, like the prenatal choline supplemented rats, were significantly less immobile during the second swim session.

3.2 Behavior in the forced swim tests is not accounted for by differences in learning and memory

Another interpretation of the forced swim data is that the rats that displayed less immobility during the second swim session may have had impaired memory for the previous swim session. For this reason, learning and memory was assessed in the water maze. This served to establish learning rates in the different diet groups with a similarly aversive swimming experience. All rats displayed excellent learning in the water maze, good retention of the spatial location of the platform 24 hours and 7 days later, and learned a new platform position during reversal training. These data clearly demonstrate that the results of the forced swim test are not accounted for by memory deficits in the prenatally and postnatally supplemented rats. In addition, it is well established in the literature that pre- and postnatal choline supplementation enhance learning and memory in rats (reviewed in Meck & Williams, 2003). It should be noted that we did not observe enhanced spatial learning in the present study. The previously reported effects are mainly evident when challenging tasks are employed, like the 12-arm radial arm maze. Thus, it was not surprising that memory-enhancing effects of choline supplementation like those previously reported were not observed in the present study as rats are quite good at the simple water maze task that was used to assess spatial reference memory and reversal learning. Nonetheless, these data allowed us to rule out differences in learning and memory deficits or reactions to the water and swimming as contributing factors to the other behavioral measures.

3.3 Choline supplementation reduces anxiety-like behavior in the open field

The findings that pre- and postnatal, but not adult, choline supplementation induced antidepressant-like effects in the forced swim test was well complemented by the results of the open field test. This test permitted the assessment of general activity levels in a large arena while simultaneously evaluating the anxiety-like characteristics of the rats' behaviors.

Thus, as mentioned previously, the test confirmed that the different diet manipulations did not produce differences in overall activity levels. However, in the rats treated with choline prenatally or postnatally, there were notable decreases in anxiety-like behaviors. This was observed on two measures: these rats explored more of the entire arena during the test and they spent more time in the central, anxiety-provoking region than the control or adult-supplemented rats. Given the inherent difficulties in establishing depressive symptoms in non-human animals and given the preponderance of anxiety symptoms in association with human depression (Aina & Susman, 2006), anxiety measures are a widely used index in rodent models of depression. In addition, increases in anxiety induced in these models are effectively reversed using antidepressant drugs (Bespalov et al., 2010; for discussion see Duman & Monteggia, 2006). In light of this, the present results of both antidepressant effects in the forced swim test and anxiolytic effects in the open field in rats given choline supplementation during developmental sensitive periods, but not those given it as adults, are together compelling evidence for the hypothesis that this nutrient exerts organizational changes in neural systems that are protective against potential triggers for psychological disorders. In humans, a clinical diagnosis of 'anxious depression' is often associated with poor responses to pharmacotherapies and the need for combined or augmented therapies is high (Rao & Zisook, 2009; also see Aina & Susman, 2006). Taken together, the patterns in the human clinical literature and the findings of the present study strongly suggest that attention to dietary features like choline levels, particularly in children displaying depressive symptoms or at risk for them, is warranted.

3.4 Choline may exert its antidepressant-like effects by enhancing adult hippocampal neurogenesis

It is not entirely clear how choline may be acting during brain development to exert the antidepressant effects reported in this paper but our previous work emphasized a possible neural mechanism: We previously reported a remarkable doubling of the numbers of newly born neurons in the hippocampus of adult rats treated *in utero* with choline supplementation (Glenn et al., 2007). In light of many compelling demonstrations in the field of the strong relation (e.g. Yau et al., 2011; reviewed in Kempermann & Kronenberg, 2003) and even the necessity (Santarelli et al., 2003; Perera et al., 2011) for adult hippocampal neurogenesis in depression and antidepressant actions, this is an excellent candidate mechanism by which choline exerts antidepressant properties. We do not presently have evidence of whether or not adult hippocampal neurogenesis is altered by giving rats postnatal or adult choline supplementation for the durations that were used in the present study. Determining whether postnatal, but perhaps not adult, choline supplementation increases adult neurogenesis would be particularly informative. Wong-Goodrich et al. (2008b) reported significant increases in cell proliferation in the dentate gyrus of the hippocampus, marked by the cell division marker bromodeoxyuridine (BrdU), following adult choline supplementation. Although neurogenesis was not examined in that study, it is possible, based on their findings, that even adult choline supplementation may increase neurogenesis.

It is intriguing, then, that there was evidence from the present study that adult choline supplementation had some antidepressant and anxiolytic effects: rats in this diet group spent more time in the center of the field than control rats, despite being less active overall, and they took longer to become immobile on the second forced swim test. Wong-Goodrich et al. (2008b) fed their rats a choline-supplemented diet for 16 weeks prior to assessing hippocampal cell proliferation, nearly five times longer than the duration of choline supplementation that was used in the present study. The present results specifically indicate that the duration of choline supplementation needed during prenatal and postnatal development (12 and 25 days respectively in the present study) is sufficient to induce antidepressant effects on behavior, while 25 days of choline supplementation in adulthood is

not. Thus, it is possible that a longer duration of adult choline supplementation may have more robust behavioral effects. Further work on dissociating the organizational and activational mechanisms of choline supplementation is warranted and ongoing.

3.5 Choline may exert its antidepressant-like effects by enhancing neurotrophic activity

Another key mechanism for the antidepressant properties of developmental choline supplementation, in addition to or contributing to its neurogenic properties, may be its actions on neurotrophic systems. Like adult hippocampal neurogenesis, growth factors, like BDNF, are also decreased in rodent models of depression and increased by antidepressants (reviewed in Duman, 2004; Hashimoto et al., 2004; Nagahara & Tuszynski, 2011). In fact, central administration of BDNF exerts antidepressant effects akin to those observed with antidepressant drugs (Siuciak et al., 1997). Similarly, genetic manipulations that compromise BDNF functioning induce depression-like symptoms in mice (Monteggia et al., 2007) and humans (for discussions and reviews see Duman & Monteggia, 2006; Tsai et al., 2010; Verhagen et al., 2010). In our previous work we found that levels of BDNF in the adult hippocampus were increased with prenatal choline supplementation (Glenn et al., 2007; 2008; also see Nag et al., 2007; and Napoli et al., 2008). Thus, increases in growth factor expression may underlie the enhanced production and/or survival of new neurons in the hippocampus, particularly in the face of environmental triggers that normally diminish this process. It is also possible that choline supplementation during development may modulate the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis such that exposure to glucocorticoid hormones, which decrease adult hippocampal neurogenesis, may be reduced. We previously found little change in basal levels of the glucocorticoid hormone, corticosterone, or the reactivity of the HPA axis to acute restraint stress with prenatal choline supplementation (Glenn et al., 2008). However, the possibility that some of the effects observed in the present study may be accounted for by changes in HPA function cannot be ruled out as the rats in our previous study were assessed before the onset of puberty and it is not known whether stress reactivity is affected by the present diet manipulations in adult rats.

3.6 Possible genomic and epigenomic mechanisms of dietary choline supplementation

The antidepressant properties of choline supplementation reported here are further supported by converging evidence from the fields of choline and depression research on possible epigenetic factors. Choline, through its conversion to betaine, is an essential source of methyl groups needed for methyl reactions in the body. In pregnant mice consuming a methyl-rich diet that included supplemental choline, the phenotype of offspring in coat color and body mass composition were altered epigenetically by increased methylation of the viable yellow agouti gene (Waterland & Jirtle, 2003). On the other hand, choline supplementation led to an overall reduction in global DNA methylation in brain suggesting that there are more complex effects at play (Kovacheva et al., 2007). For example, choline modulates levels of methylation enzymes (Kovacheva et al., 2007) and alters histone function (Davison et al., 2009; Mehedint et al., 2010). The latter findings are particularly intriguing in light of the well-known antidepressant properties of inhibitors of histone deacetylase, an enzyme that leads to the removal of acetyl groups from histones. When histones are deacetylated they are less permissive to gene transcription and therefore preventing deacetylation can promote gene transcription. This is a provocative target for future investigations into the mechanisms of choline's antidepressant properties, demonstrated in the present report, but also its more general neuroprotective properties, which are well-documented (Glenn et al., 2008; Guo-Ross et al., 2003; Holmes et al., 2003; Moon et al., 2010; Nag & Berger-Sweeney, 2007; Thomas et al., 2000; 2007; Wong-Goodrich et al., 2008a; 2011).

Work on choline's genomic action also reveals an increased need for choline in populations with relatively common genetic polymorphisms that render them less able to make choline endogenously or to utilize it efficiently (Zeisel, 2007; 2011). There is also an estrogen promoter region on the gene that codes for the endogenous synthesis of choline that leads to an increased need for choline intake in females depending on their estrogen status (Zeisel 2008; 2011). These genetic findings point to the possibility that there may be vulnerable populations for which dietary choline intake could be a key contributor or risk factor for psychopathology and thus a key avenue for therapy. Furthermore, we previously found that prenatal choline deficiency prevented a normal enrichment-induced increase in adult neurogenesis in female rats (Glenn et al., 2007). This finding is of particular interest and relevance because it suggests that choline deficiency, arising from diet practices or a genetic condition, may reduce the potential for antidepressants to increase neurogenesis and may thereby interfere with their effectiveness.

3.7 Summary

In summary, the results of the present study are a compelling demonstration that the amount of dietary choline during prenatal or postnatal developmental periods in female rats confers a substantive antidepressant-like effect as indexed using the forced swim test. This test was developed to evaluate the efficacy of novel antidepressants and has excellent predictive validity for the therapeutic potential of new drugs. Quite remarkably, the rats that were supplemented with choline during development in the present study displayed reduced immobility in the forced swim test that was of a strikingly similar magnitude to that which results when rats are given antidepressants before the assessment. The rats in this study were not under the influence of additional choline when tested and in fact the rats that were fed choline supplemented diets only in adulthood were under the influence of additional choline and did not differ significantly from control rats. The results of the present study also confirm that there were no differences in learning or memory that could account for these effects and they were additionally supported by reductions in anxiety-like behaviors assessed in the open field test. Based on past work, it is likely that these behavioral effects are the result of increased adult hippocampal neurogenesis and neurotrophic function. The possibility that these neural changes are the result of epigenetic modifications to DNA methylation patterns and histone functioning are exciting new hypotheses that have the potential to not only emphasize the powerful nature of nutrients to exert potent neuroprotective effects but may also help shed light on the neurobiological mechanisms of psychological disorders, like depression.

4. Experimental Procedure

4.1 Rats and Choline Diets

All rats used for this study were housed in individually ventilated clear polycarbonate cages (30.8 × 30.8 × 18.7 cm; Thoren Caging Systems, Hazleton, PA). The colony was maintained on a 12:12 h light-dark cycle with lights on at 08:00 h; the colony temperature was 21±2 °C with 40–60% humidity. Twenty timed-pregnant Sprague-Dawley rat dams (CD strain, Charles River Breeders, Raleigh, NC) arrived in the colony on day nine of gestation (embryonic day; ED 9). Upon arrival in the colony, dams were housed individually and given *ad libitum* access to water and the choline-sufficient diet (AIN76A with 1.1 g/kg choline chloride substituted for choline bitartrate; Dyets Inc. Bethlehem, PA). From ED 10 until birth, some of the dams (n=6) were placed on the same synthetic diet but with supplemented choline content (SUP; AIN76A with 5 g/kg choline chloride); the other 14 dams remained on the choline-sufficient diet (See Fig. 1). On PD1–2 litters were collected and all pups were permanently marked with a toe clip to indicate their choline diet condition. New mixed litters containing 10 pups of males and females from choline-

sufficient and -supplemented dams were assembled and given to choline-sufficient fed foster mothers for rearing. On PD24 female pups were weaned into small social groups of 4 rats, all from the same prenatal diet condition. On PD50, the rats were divided into same-diet pairs and housed in this way for the remainder of the study.

A total of 40 females served as subjects and they were divided into four groups, three of which received supplemental choline in their diets at different points in the lifespan and one control group (see Fig.1). One was comprised of rats that were the offspring of choline-supplemented dams (PRE-SUP; n=12) and they were fed the choline-sufficient diet for the remainder of the study. The three other diet groups were the offspring of choline-sufficient dams. One group was placed on the choline-supplemented diet during the three weeks after weaning, specifically on PD25–50 (POST-SUP; n=8). A second group of rats from choline-sufficient dams were placed on the choline supplemented diet on PD75 (ADULT-SUP; n=8) and remained on it until the end of the study. The third group of rats from the choline-sufficient fed dams (and the fourth condition in the experiment) served as the control group and were kept on the choline-sufficient diet for the entire study (CONTROL; n=12). Thus, rats from the three choline supplemented groups were only fed that diet during the prescribed period (prenatal, postnatal, and adult) and at all other times in the study they were fed the CONTROL (choline sufficient) diet. Behavioral assays commenced on PD 100, at which time the ADULT-SUP rats had been on the supplemented diet for 25 days.

The ages used in the present study were selected to capture different periods of development. The effects of prenatal and, to a lesser extent, postnatal choline supplementation are established as sensitive periods for choline's effects on brain and behavior (reviewed in Meck & Williams, 2003; also see Meck et al., 2007). Less is known about choline supplementation in adult rats and we thus sought mainly to ensure that rats were fully adult before manipulating choline levels in their diet. Thus this requirement was clearly fulfilled by beginning the adult supplementation of choline on PD 75.

4.2 Estrous cyclicity

The phase of the estrous cycle in the female rats was assessed at regular intervals throughout the course of the study and on all training and test days of the tasks outlined below. We detected no evidence that the different diet conditions impacted puberty onset, which we indexed by vaginal opening, or the start of regular estrous cycles. Cycle phase was identified by obtaining a vaginal cell sample with a moist cotton-tipped applicator. The sample was smeared on a slide and examined microscopically under 100× magnification. An abundance of cornified epithelial cells was considered indicative of estrus while an abundance of nucleated cells was considered indicative of proestrus. The majority of the female rats in the study displayed regular cyclicity over a 4–5 day period.

4.3 Open Field: Activity and Anxiety

Activity levels and anxiety-like behavior were assessed during a 5-minute open field test. The open field apparatus was a 100 × 100 cm arena made of wood and painted black. Rats were placed in the field one at a time and could freely explore the space during the test. Their movements during the test were tracked and summarized using a computerized tracking system (HVS Image Ltd, Hampton, UK). The primary dependent measure of activity was the percentage of time during the test that the tracking software detected movement by the rat. The primary dependent measure of anxiety was the percentage of time during the test that the rat spent in the center of the field—during analysis of the rats' movements the computer program overlaid a 4×4 grid on the open field and thus 'center' was movement in any of the 4 squares in the center of the 4×4 grid. As a secondary measure of anxiety we measured exploration as the percentage of the 16 squares the rat entered

during the test. To minimize odor cues from prior tests, the field was cleaned thoroughly between rats using the same disinfectant employed to sanitize the colony and cages (Odoban, Clean Control Corp. Warner Robins, GA).

4.4 Water Maze: Spatial Learning and Memory

One week after open field testing, rats began training on a spatial reference memory task in a water maze. The water maze was a circular pool, 153 cm in diameter and 70 cm deep, constructed of galvanized metal and painted black. It was situated in a large room containing a variety of extramaze cues. The pool was filled with water to a depth of approximately 42 cm and made opaque through the addition of non-toxic black tempura paint. A circular escape platform was 40 cm high with a 10 cm diameter; it was thus submerged approximately 2 cm below the surface of the water. All swim trials were tracked and summarized using a computerized system (HVS Image). On each of 3 days of training on the spatial reference memory task rats received 4 trials in the pool. On each trial a rat had 60 seconds to search the pool for the hidden platform and escape onto it. A trial was terminated once a rat's front and hind paws were on the platform, or after 60 seconds if the rat did not find the platform—in this case the rat was guided to it by hand by the experimenter. Once on the platform, rats remained there for 10 seconds before being removed from the pool and returned to a holding cage within the water maze room. Rats were not able to see the room from the holding cage and they remained there between trials; the inter-trial interval was between 5 and 10 minutes. Rats were tested in groups of 6–8 and all rats received one trial before the first rat received its next trial. On each trial rats were released into the pool from one of 4 different start positions around the perimeter, arbitrarily designated as the cardinal points of N, S, W, and E. Each release point was used once per day and the order on each day was randomly determined. The primary dependent measure on training trials was the latency for rats to reach and escape onto the platform which was hidden below the surface of the water.

On the last of day of training on the spatial reference memory task a probe trial was inserted between the first two and the last two trials. On a probe trial the platform was removed from the pool and the search pattern of each rat was tracked and analyzed to determine the extent to which they were relying on a spatial strategy to locate the platform. The primary dependent measure on the probe trial was the percentage of time the rats spent in the quadrant that contained the platform on training trials (the 'target' quadrant). Rats that were searching the pool randomly would be expected to spend about 25% of its total swim time in the target quadrant, thus as values increase above 25% for that one quadrant the more the rat is displaying a nonrandom bias toward it. This probe trial was conducted close to the time of testing so that we could gauge the extent of learning of the spatial location of the platform from the previous days of training.

To assess long-term memory of the platform's location we conducted a second probe trial a week later and analyzed rats' bias towards the target quadrant in the same way as with the first probe trial, given during training. After the second probe trial, rats were tested on a reversal task for one day of 4 trials. On this task the platform was positioned in the quadrant opposite to the one used for the initial training. Rats' ability to learn the new position was indexed by their latencies to find the platform in the new location over the 4 trials.

4.5 Forced Swim Test: Behavioral Despair

One week after the last day in the water maze, we began conducting the forced swim test of behavioral despair. This test was conducted using a glass cylinder that was 25 cm in diameter and 75 cm high. The cylinder was filled with 40°C water to a height of 40 cm. All rats received two experiences in the water column as described by Porsolt et al., 1978. We

referred to the first experience as the 'induction phase'. In this phase the rat was placed in the cylinder of water, from which there was no escape, for 10 minutes. This experience is intended to induce a state of despair in the rats and the extent to which it is psychologically traumatic is then indexed in the second experience in the water column. We classified the second experience as the 'assessment phase' and it was conducted 48 hours after the first phase and lasted 5 minutes. In the forced swim test an altered mood state, or despair, is generally expected to appear in the assessment phase and is indexed by the emergence and extent of immobility displayed by the rats. The criterion for immobility was a minimum of 3 seconds without movement except for small movements made by the rat to keep its head above the water. All swim sessions were videotaped and the behavior of rats in the water columns was coded from the digital records by an experimenter that was blind to the diet condition of the rats.

For both the induction and assessment phases of the test, we recorded the latency to the first instance of immobility meeting the criteria; this measure allowed us to evaluate how quickly rats 'gave up' in each phase of the test. We then recorded the amount of time each rat spent immobile during each minute of each test; this allowed us to study the progression of immobility over the course of each test. Using the data from each minute of each phase of the test we were also able to obtain the total amount of time rats spent immobile during induction and assessment. Additional measures included the number of dives made and the number of fecal boli at the end of the swim session.

4.6 Statistical Analyses

Unless indicated otherwise, the effect of the independent variable of diet on each of the dependent measures described above was assessed using one-way analyses of variance (ANOVA). Following a statistically significant ANOVA result, each of the 3 choline-supplemented groups were compared to the control-fed group using post hoc Dunnett tests. Planned comparisons were conducted where necessary (as in the case of non-significant main effects or interactions) with the guiding hypothesis that choline-supplemented rats would display less anxiety-like behavior, better learning and memory, and less despair than control-fed rats. Analyses of co-variance (ANCOVA) were also conducted on dependent measures with the phase of estrous cycle as the co-variant. However, no difference from the initial ANOVA results was found and these are reported below. One sample t-tests were used to compare the search time percentages of each group to that which would be expected if the 4 pool quadrants were being searched randomly (25%). Data met the requirements of normal distribution and thus met the requirements of parametric tests. All statistical tests were evaluated for significance with an α level of 0.05.

Highlights

- Supplemental dietary choline was given to female rats at different points in lifespan
- Supplemental choline during development reduced behavioral despair in adults
- Supplemental choline during development reduced anxiety in an open field in adults
- Supplemental choline in adults had little effect on anxious or depressive behaviors
- Differences in despair and anxiety are not explained by differences in learning

Acknowledgments

The authors would like to acknowledge the helpful comments of Lauren Lessing and Amanda Kimball on an earlier version of this manuscript and the editorial assistance of Meghan Tedoldi and Joseph-Daniel Saporta. The project described was supported in part by Award Number P20RR016463 from the National Center for Research Resources to MJG.

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Fig. 1.

Experimental design and timeline. Pregnant dams arrived in the colony on embryonic day (ED) 9 and a subset were fed a choline supplemented diet (offspring of these dams were PRE-SUP) while the remainder consumed the control diet. Day of birth was postnatal (PD) 0 and cross-fostering to control-fed dams occurred within 1–2 days of birth. Rats were weaned on PD24 and a second group of subjects were fed a choline supplemented diet from PD25–50 (POST-SUP). A third group received choline supplementation beginning on PD75 and extending until the study end (ADULT-SUP). Behavioral testing began on PD100.

Fig. 2.

Behaviors of rats in the open field as a function of dietary choline conditions. The percentage of the total test time moving in the field (A) was used to gauge the general activity levels of the rats; percentage of the field explored (B) and percentage of time spent in the center of the field (C) was used to gauge anxious-like behaviors in the rats. # is $p < 0.05$ compared to PRE-SUP; * is $p < 0.05$ compared to CONTROL. Bars are $\pm$ SEM.

Fig. 3.

Spatial learning and memory in the water maze. Panel A shows the latency to find and escape onto the hidden platform averaged over 4 trials on each of 3 training days. The percentage of time rats spent in the quadrant of the pool that contained the platform on training trials on probe tests conducted on the last day of training (B) and a week after training (C) is shown as a function of the diet conditions. In (B) and (C) the dashed line indicates the amount of time that would be spent in that quadrant in rats randomly searching the pool (25%). * is $p < 0.05$ compared to chance levels. Bars are $\pm$ SEM.

Fig. 4.

Response of rats to the first forced swimming exposure (induction) as a function of dietary choline levels. Top panels show the latency to the first instance of immobility (A) and the total time amount spent immobile during the 10-minute test (B) as a function of dietary choline conditions. The bottom panel shows the time spent immobile during each minute of the 10-minute session as a function of dietary choline conditions. Arrows indicate patterns with less immobility in PRE-SUP rats compared to CONTROL. Bars are $\pm$ SEM.

Fig. 5. Response of rats to the second forced swimming exposure (assessment) as a function of dietary choline levels. Top panels show the latency to the first instance of immobility (A) and the total time amount spent immobile during the 5-minute test (B) as a function of dietary choline conditions. The bottom panel shows the time spent immobile during each minute of the 5-minute session as a function of dietary choline conditions. * is $p < 0.05$ compared to CONTROL. Bars are $\pm$ SEM.