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Increased homocysteine levels associated with sex and stress in the learned helplessness model of depression

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Abstract

Elevated levels of homocysteine (Hcy) have been associated with major depressive (MD) illness. As human females show a higher predisposition towards depression, this study examined how Hcy levels in rats are affected by sex and estrous cycle in the learned helplessness (LH) model of depression. Male and female rats in either estrus or diestrus were subjected to LH, with intervals of 4 days between the two stress tests and between tests and sacrifice, in order to accommodate the female estrous cycle. No differences were found in LH behavior between males and females at either estrous phase. Control Hcy levels were significantly lower in females than in males (−36%, $P<.001$), with no further differences between estrous and diestrus phases in females. Stress exposure increased plasma Hcy by approximately 26% in females, both in estrus and diestrus, but not in males. However, when behavioral responses to stress were considered, no association was found between increased Hcy levels and propensity to develop helpless behavior. Therefore, while male rats have higher basal Hcy levels than females, females appear to be more vulnerable than males to stress-induced elevations in Hcy, although this did not correlate with behavioral responses to stress. Neither was this vulnerability influenced by estrous phase. These results imply that both stress and sex should be considered as risk factors for increased plasma Hcy.

Keywords: Stress; Sex; Estrous cycle; Major depression

1. Introduction

A well-known and consistent phenomenon in psychiatric epidemiology is the tendency for females to be more likely to develop major depression compared to males (Kessler et al., 1993; Weissman et al., 1993). Such a difference may be attributed to gonadal hormones and/or fluctuations in their changing levels. This is supported by the observations that prevalence to depression in males and females is the same when women are prepubertal and postmenopausal (Hale and Cochran, 1983), but prevalence doubles when females are in their reproductive years (Angold and Worthman, 1993; Kessler et al., 1993). Furthermore, over one third of women with major depression report premenstrual increases in depressive symptomatology (Endicott, 1993; Yonkers and White, 1992), suggesting a role of the menstrual cycle and fluctuating hormones in depression.

Homocysteine (Hcy) is an intermediate in the methionine metabolic pathway that can be metabolized via transsulfuration to cystathionine using vitamin B_6 as a cofactor or via folate-dependent remethylation to methionine, which is subsequently converted to S-adenosylmethionine (SAMe). The latter is a ubiquitous methyl donor, which in controlled studies has been shown to exhibit antidepressant properties (Bell et al., 1988; Kagan et al., 1990; Fava et al., 1997; Delle Chiaie et al., 2002). Hcy levels are known to be affected by dietary factors such as methionine, by deficiencies in vitamins B_{12}, B_6, and folate, and possibly by non-dietary factors such as stress (Stoney, 1999; but see also Farag et al., 2003). High concentrations of plasma Hcy or hyperhomocysteinemia have been associated with major depressive (MD) disorder. Thus, it has been found that MD patients with elevated Hcy levels have generally higher Hamilton scores relative to MD patients with normal Hcy levels (Bottiglieri et al., 2000). Elevated total plasma Hcy

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concentrations (>12.0 μmol/l) have been reported in 20–50% of depressed patients (Fava et al., 1997; Bottiglieri et al., 2000). Further, Hcy is a sensitive marker of functional deficiency of either folate or vitamin B_{12} (Stabler et al., 1988), and there is evidence of a high incidence of folate deficiency in major depression (Crellin et al., 1993) and of significant antidepressant effects of folate supplementation (Godfrey et al., 1990).

One study has reported that plasma Hcy concentrations are dependent on menstrual cycle phase, hence reinforcing the association between estrogen status and Hcy levels (De Cree et al., 1999). Another study, however, failed to find significant Hcy changes across the menstrual cycle (Merki-Feld et al., 2000). There are indications that estrogen replacement therapy may provide beneficial antidepressant effects in perimenopausal (Rasgon et al., 2002) and postmenopausal women (Miller et al., 2002) and in addition may help reduce total Hcy levels in postmenopausal women (Zmuda et al., 1997). Further studies of sex/gonadal differences in animal models of depression may shed light on the precise role of elevated Hcy levels in depressive conditions. A recent study of estrous cycle in female rodents in the learned helplessness (LH) paradigm has shown significant behavioral differences when two phases of estrous cycle, estrus, and diestrus were examined (Jenkins et al., 2001). As these two phases differ in the amount of time since release of estrogen and progesterone, they may potentially affect circulating levels of Hcy. The present study tested this hypothesis in the LH model, a well-validated and accepted model of major depression (Willner, 1986, 1991). This model centers on stress-induced behavioral depression, in which prior exposure to inescapable stress produces deficits in escape testing. The aim of the present study was therefore to ascertain how Hcy plasma levels in rodents are affected by sex and estrous cycle in this model of depression.

2. Methods

2.1. Animals

Age-matched Sprague–Dawley rats (Charles River Laboratories, Montreal, Quebec), female (n=58) and male (n=32), were housed in same-sex pairs, with free access to water and standard rat chow (Purina Rodent Diet 5001 containing 5.9 ppm of folic acid, 22 μg/kg of vitamin B_{12}, and 6.0 ppm of pyridoxine) at all times. Animals were kept on a 12:12-h light/dark cycle at a room temperature of 21±1 °C. Body weights at time of arrival ranged from 176 to 200 g (52–63 days old) for females and 226 to 250 g (50–52 days old) for males. All animals were weighed and handled daily for an acclimatization period of 1.5 weeks and throughout the subsequent 2-week test period to ensure normal weight gain progression. Daily vaginal smears via lavage using a blunted Eppendorf tip with 20 μl of 0.9% saline were obtained from all females, including controls. Slide-plated smears were examined using a Nikon Eclipse E600 microscope and classified according to estrous phase (Maeda et al., 2002). Only females showing two to three consistent estrous cycles were used for further study. All animal procedures were approved by the institutional ACC and were in compliance with the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Stress procedures

Animals were segregated according to sex and estrous cycle (diestrus and estrus). Five males, eight females in estrus, and eight females in diestrus were designated as cage controls and were not exposed to footshock stress. All remaining animals, namely 18 males, 20 females in estrus, and 18 females in diestrus, were exposed to LH procedures. The LH procedure involves one inescapable and one escapable shock session, which are usually separated by a 24-h period (Drugan et al., 1982; Petty et al., 1992). In this scenario, females would inevitably be in the next phase of the cycle 24 h later during the escapable shock session and further into the following phase at sacrifice. To avoid this and maintain all testing and sacrifice at the same estrous phase, this schedule was changed to a 4-day rather than a 1-day rest period between sessions. This schedule was determined by monitoring the estrous cycle time patterns for the given colony for at least three full normal cycles prior to start of the experiment.

On Day 1, animals were placed in sound-attenuated operant boxes (Med Associates, Georgia, VT) where they received inescapable shock with lights off. Shock was delivered as a scrambled pulsed 0.8 mA current through metal floor bars. Both shock duration and intertrial intervals were randomly varied (1.5–60 and 1–30 s, respectively), for a total shock exposure of 25 or 30 min.

On Day 5, animals were placed in the operant boxes and given exactly 15 trials of escapable shock, each lasting for a maximum 60 s duration, with a fixed intertrial interval of 24 s. Initiation of shock (0.8 mA) was accompanied by a white light placed directly above a lever, which when pressed terminated the shock on a fixed ratio-1 (FR-1) schedule and subsequently turned off the cue light. A house light outside the immediate chamber was kept on during the entire trial. A bar press within the first 20 s of initiation was recorded as an escape response. A response between 20 and 60 s was classified as failure to escape. After 60 s, the shock was automatically terminated and the trial counted as a failure. An escape response resulted in termination of the shock for the remainder of the 60-s trial and then was followed by the 24-s intertrial period. Escape performance and latency to escape were recorded for each animal over the 15 trials. Animals were classified as LH if they failed to escape in 10 or more of the 15 trials. Conversely, rats that failed to escape in 5 or less of the 15 trials were termed resistant or nonlearned helpless (nLH) (Edwards et al., 1986). Animals

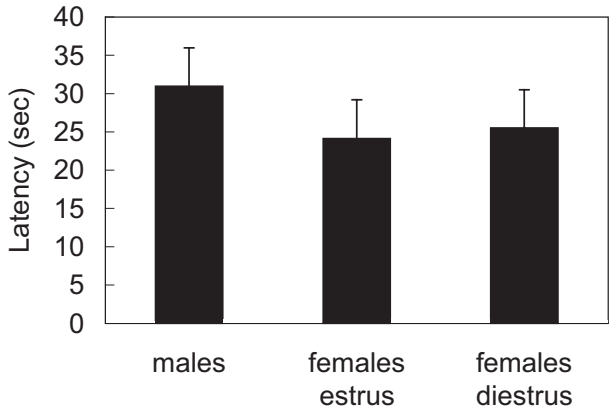

Fig. 1. Latency to escape averaged over 15 trials in the escapable shock session. Each bar is a mean (±S.E.M.) of 14 animals. No significant differences were found between groups.

that failed 5–10 times were not used for subsequent plasma analyses.

2.3. Blood collection and Hcy assays

Animals [females in estrous: LH (n=6), nLH (n=8), control (n=8); females in diestrus: LH (n=6), nLH (n=8), control (n=8); males: LH (n=6), nLH (n=8), controls (n=5)] were sacrificed on Day 9 (4 days after the escapable shock trial session) via decapitation. Trunk blood was collected into heparinized vials and centrifuged. Resulting plasma was removed from each sample and kept frozen at −80 °C for Hcy analysis. Plasma total Hcy values were determined by high performance liquid chromatography with fluorimetric detection and isocratic elution (Pfeiffer et al., 1999). This methodology involves three steps, namely, reduction of thiol groups using TCEP [tris(CarboxyEthyl)-Phosphine], protein precipitation, and derivatization with SBD-F (7-fluorobenzene-2-oxy-1,3-diazolic-4-ammonium sulfate). The HPLC system used was a Shimadzu apparatus with an SIL-10ADvp automatic sample injector and an RF-10AXL fluorescence detector. Chromatographic separation was performed using a C18 model Shim-pack CLC-ODS column (4.6×150 mm, with 5.0 μm microparticles). The fluorescence of the separated compounds was detected with a detector adjusted for excitation at 385 nm and emission at 515 nm. Total Hcy content was calculated with a calibration curve using known Hcy concentrations and cysteamine as the internal standard.

2.4. Data analysis

Behavioral latencies and Hcy levels were analyzed by two-way analyses of variance (ANOVA) followed, where appropriate, by independent t tests. Chi-square tests were used to analyze frequency data.

3. Results

3.1. LH behavior

As illustrated in Fig. 1, mean latencies to escape shock in the 15-trial session were not statistically different among males, females in estrus, and females in diestrus [$F(2,47)$= 0.43, P=.65]. When a number of escape failures were used to categorize animals as helpless (LH), nonhelpless (nLH), or neither, as defined above, 44% of the males, 25% of females in estrus, and 33% of females in diestrus fell in the LH category; whereas in each of these sex groups, 44%, 65%, and 61%, respectively, were classified as nLH. A chi-square test indicated no significant differences in the fre-

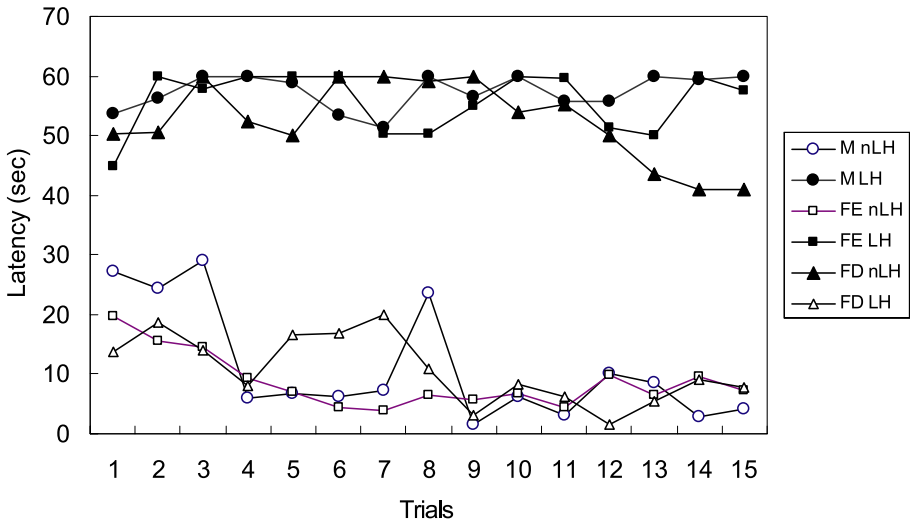

Fig. 2. Latency to escape over 15 trials of escapable shock in males, females in estrus, and females in diestrus. Each point is a mean of 5–9 animals (see text); error bars are omitted for clarity. Filled dots indicate animals categorized as learned helpless (LH), and empty dots indicate nLH. M: males; FE: females in estrus; FD: females in diestrus. No significant differences were found among gender groups within each of two behavioral categories.

quency of these behavioral outcomes among males, females in estrus, and females in diestrus (χ^2=4.10, *df*=4, *P*=.39).

When escape latencies were examined over individual trials (Fig. 2), no main effect of sex/estrous phase was detected [$F(2,44)=0.75$, $P=.47$]. A strong significant overall difference between helpless and nonhelpless animals was confirmed [$F(1,44)=63.84$, $P<.001$], but there was no interaction between sex/estrous phase and helplessness [$F(2,44)=0.84$, $P=.435$]. Further comparisons between males and females (irrespective of estrous phase) likewise revealed no significant differences ($t=1.03$, $P=.32$).

Together, these observations clearly indicate that under the present conditions, sex and estrous phase did not influence vulnerability towards helplessness.

3.2. Hcy plasma concentrations

Hcy levels are shown in Fig. 3. A 3×3-way ANOVA revealed a significant main effect of sex [$F(2,55)=26.40$, $P<.001$], a significant main effect of helplessness [$F(2,55)=5.89$, $P=.005$], and a significant Sex×Helplessness interaction [$F(4,55)=3.44$, $P=.014$].

Further analyses of sex effects (Fig. 3) confirmed that in cage control groups, Hcy levels were significantly higher in males compared to females in estrus ($t=10.44$, $P<.0001$) or diestrus ($t=6.70$, $P<.0001$), whereas the two female groups did not differ among themselves ($P>.05$). Among male groups, pairwise comparisons revealed no significant differences among LH, nLH, and control groups (all $P>.05$). Female results presented a different trend. Significant differences in Hcy were found between shock-exposed females and controls, and this was seen both in estrus ($t=4.36$, $P=.003$) and diestrus groups ($t=2.96$, $P=.007$). However, behavioral outcome in stressed animals had no further effect on Hcy levels in females, as no differences were found between LH versus nLH groups in either estrus ($t=0.1$, $P=.92$) or diestrus ($t=0.67$, $P=.52$).

When male and female groups were combined, pairwise tests confirmed significant differences in Hcy levels between LH and controls ($t=2.48$, $P=.018$) and between nLH and controls ($t=3.13$, $P=.003$) but revealed no significant difference between LH and nLH groups ($t=0.39$, $P=.70$), thus confirming that behavioral outcome (i.e., LH vs. nLH) had no overall effect on Hcy levels.

In summary, under control conditions, males were found to have significantly higher levels of Hcy compared to females. Footshock stress induced significant increases in Hcy levels in females, irrespective of estrous phase, but not in males.

4. Discussion

4.1. Learned helplessness

In the present study, no differences in propensity towards LH were found among males, females in estrus, and females in diestrus. Therefore, while the LH model reproduces important features of depressive conditions in humans (Willner, 1986), it does not appear to model the higher predisposition towards developing depression observed in women compared to men.

Studies that have used models of depression to observe sex differences in propensity towards developing depressive-like behavior have produced inconsistent results. Jenkins et al. (2001) found no significant differences between males and females in FR-2 escape latencies using the LH paradigm, similar to the present findings using FR-1 escape latencies. An earlier study using the LH model found lower escape latencies in females compared to males (Steenbergen et al., 1989). Studies that examined estrous phase and depressive behavior have also shown inconsistencies. Jenkins et al. (2001) reported increased escape latency in diestrus females compared to estrus using the LH paradigm, a finding consistent with another study that reported an increased immobility in the forced swim test model during diestrus compared to estrus (Maravan et al., 1996). These effects were not seen in the present study. Our current

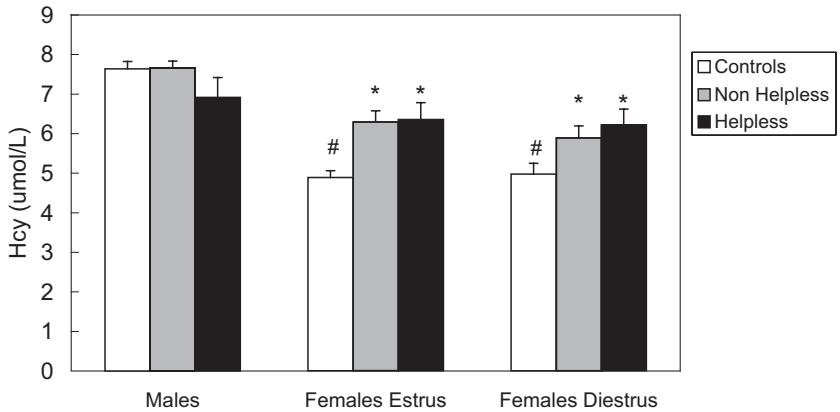

Fig. 3. Total plasma Hcy levels (μmol/l) in males, females in estrus, and females in diestrus. Each bar represents a mean (±S.E.M.) of 5–9 animals (see text). *Significantly different ($P<.01$) from the respective nonstressed control group; #significantly different from the male nonstressed control group ($P<.001$).

findings are however in accordance with an earlier study that reported no difference in the duration of immobility in the forced swim stress test between female rats during different stages of the estrous cycle (Alonso et al., 1991).

The reasons for these inconsistencies are not clear. One of the methodological differences between the present study and the Jenkins et al. (2001) study is the interval between inescapable and escapable shock sessions. We used a 4-day interval compared to Jenkins et al. (2001) who used an interval of 1 h. The more commonly used interval of 24 h between inescapable and escapable stress testing was altered in both studies in order to accommodate the female estrous cycle. Our study, by testing in same phase of the following cycle, may have measured escape behavior at a slightly different part of the phase compared to when the inescapable testing had occurred. Even within the same cycle, levels of gonadal hormones rise or fall and may possibly influence behavior. On the other hand, we reasoned that a 4-day schedule may be useful for studies aimed at examining biochemical changes across different phases of the estrous cycle since changes seen immediately poststress may be heavily influenced by generalized stress as opposed to LH behavior.

This study has shown that a 4-day interval does not influence behavioral outcome, as the number of resulting LH animals coincide with the expected amount (approximately 20%) (Edwards et al., 1986). By the same token, our findings raise questions as to whether the strain or specific LH paradigm used is in fact useful to model the female predisposition towards depression. There are studies that do report increased propensity towards LH behavior in females using other strains of rats, including the congenitally helpless and the Zucker insulin resistant rats (Edwards et al., 2000). Further study in these particular strains may be more useful to model sex issues in depressive behavior.

4.2. Hcy levels

The main findings of this study were as follows: (1) Control Hcy levels were higher in males than in female rats, with no further differences between estrous and diestrus phases in females; (2) stress exposure elevated plasma Hcy in females, both in estrus and diestrus, but not in males; and (3) when behavioral responses to stress were considered, no association was found between increased Hcy levels and propensity to develop helpless behavior.

Males were found to have significantly higher levels of Hcy compared to females in either the diestrus or estrus phase, while no significant differences were seen in control Hcy levels in estrus versus diestrus. Estrogen has been hypothesized to have a protective effect against elevations in Hcy levels (El-Swefy et al., 2002; Dimitrova et al., 2002; Morris et al., 2000; Yildirir et al., 2002), and in human populations males have increased risk towards hyperhomocysteinemia (Andersson et al., 1992; Dierkes et al., 2001; Verhoef et al., 1999). We therefore expected to see higher levels of Hcy in males compared to females, and this was in fact observed in the present study. In addition, the estrus group, which has higher levels of circulating estrogen relative to diestrus, was expected to show a lowering of Hcy levels since estrogen has Hcy lowering effects. This however was not observed in this study. It may be noted however that estrus and diestrus phases do not represent the greatest net difference in estrogen levels. Estrus and diestrus were chosen for this study based on previous indications of differences in LH behavior (Jenkins et al., 2001). However, other phases such as proestrus, where circulating levels of estrogen are at their highest, may be a better phase to examine Hcy levels and how they are affected by the estrous cycle.

When animals were exposed to physical stress, females showed substantial increases in Hcy whereas male rats did not. Human studies examining the effects of stress on total Hcy levels have produced inconsistent results. One study tested the effects of acute psychological stress (10 min in duration) on Hcy levels in a sample of women and found that stress rapidly elevated Hcy levels (Stoney, 1999). A recent study however reported that psychological stress (6 min in duration), menopausal status, and oral hormone replacement therapy did not affect plasma Hcy levels in women with normal basal Hcy levels (Farag et al., 2003). This suggests that type of stressor and duration may be factors that affect Hcy levels in females, which can be a complicating variable if Hcy is used as a marker for stress. The LH paradigm does itself result in different amounts of total exposure to shock within and between behavioral groups since shock duration during escapable testing is entirely based on individual performance for escape. Despite this, we found no significant differences between males and females in total shock exposure or in behavioral responding to stress. It is thus possible that plasma Hcy levels in females may be generally more sensitive to stressful stimuli than in males. Another possibility is that males metabolize Hcy more rapidly and/or that stress-induced Hcy increases may return to basal levels faster in males than in females. Further studies incorporating different time points of plasma collection after acute stress should help explain this phenomenon. Since blood collection in live animals is stressful, serial blood sampling was avoided in this study in order to minimize stress response and focus only on behavioral response.

While plasma Hcy levels in females showed a significant response to stress, we found no association between elevations in Hcy and behavioral responses to stress. That is, plasma Hcy was equally elevated in stressed females showing helpless behavior and in those showing resistance to helplessness, irrespective of estrous phase. Thus, unlike the clinical data in depressed patients, LH was not associated with elevated Hcy levels. It is important to appreciate however that elevated Hcy is not a hallmark feature of depression and may occur in less than 30% of the depressed population (Bottiglieri et al., 2000; Fava et al.,

1997). Nonetheless, based on the results of this study, it appears that the LH paradigm does not model this particular trend in clinical populations. Interestingly, however, a recent study found that Dahl rats, which model hypertension, have a higher propensity towards developing LH behavior compared to Sprague–Dawley rats (Edwards et al., 2000). As there is accumulating clinical evidence that elevations in plasma total Hcy are increased in patients with essential hypertension (Bortolotto et al., 1999; Perry, 1999; Mendis et al., 1999), the use of hypertensive rat strains may be useful in further exploring the relationship between depressive behavior and hyperhomocysteinemia-associated disease.

In summary, female rats showed no higher propensity than males to LH, but unlike males showed significant elevations in plasma Hcy in response to stress. The precise role of Hcy in depressive conditions remains unclear, but the current findings suggest that females may be more susceptible to the Hcy-elevating effects of stress. This is of concern in view of potential cellular damage that may ensue with higher levels of Hcy (Duan et al., 2002). In addition to being an independent risk factor for cardiovascular disease, high Hcy levels may potentially elicit a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity (Kruman et al., 2002). Understanding the factors that regulate plasma Hcy levels may potentially aid in preventing these risks in a variety of conditions, including stress-related disorders.

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