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Chemotherapy drug thioTEPA exacerbates stress-induced anhedonia and corticosteroid responses but not impairment of hippocampal cell proliferation in adult mice

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Abstract

Cancer patients often suffer long-lasting affective and cognitive impairments as a result of chemotherapy treatment. Previous work in our lab has shown deficits in learning and memory and hippocampal cell proliferation in mice lasting up to 20 weeks following acute administration of thioTEPA. In this study, the effects of thioTEPA in conjunction with effects of chronic stress on depression-related behavior were examined in C57BL/6J mice, 12 weeks following thioTEPA administration. Chemotherapy-treated mice showed a diminished sucralose preference compared to controls that was further exacerbated after 2 weeks of daily restraint stress. This intensifying effect was not observed in the Porsolt forced swim test. Moreover, stress-induced corticosteroid responses were exaggerated in thioTEPA-treated mice. Cell proliferation in the dentate gyrus of the hippocampus was also impaired similarly by prior thioTEPA treatment and by daily restraint stress, with no additive effect. Results suggest that some depression-related impairments may be exacerbated by chemotherapy treatment through altered corticosteroid regulation.

Keywords

Chemotherapy; neurogenesis; depression-related behavior; corticosteroid response; stress; dentate gyrus

1. Introduction

Many cancer patients undergoing chemotherapy treatment experience chemo brain, an array of cognitive and affective impairments in memory, concentration, language, motor skills, or mood that often extend beyond the duration of the treatment for months or even years. Understandably, patients often also suffer from long-lasting psychological depression as a part of their chronic illness, with the stress of treatment, thoughts of mortality, and debilitating symptoms accompanying cancer and chemotherapy significantly increasing the prevalence of clinical depression in many patients [1] which can exacerbate cognitive deficits and diminish the overall well-being of patients [2].

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Recent studies addressing effects of chemotherapy drugs on the brain have focused on the hippocampus given its prominent role in learning and memory and on regulation of mood, and in particular on the dentate gyrus of the hippocampus, in which generation of new neurons continues into adult life, well beyond the point that neurogenesis in most other brain areas has ceased. Studies in humans have provided correlative data suggesting altered hippocampal structure as a basis for depression (and other mood disorders), a suggestion supported by a spate of more recent studies using rodent models, many of which documenting impairments of proliferation, development and/or survival of newborn cells in the hippocampus by various chemotherapy drugs (for reviews, see [3–6]).

Hippocampal neurogenesis is impacted also by other factors including psychological stress (for review, see [7]), which is thought to alter neurogenesis primarily through increased corticosteroids [8–10]. Recent studies suggest that impaired neurogenesis in turn dysregulates subsequent corticosteroid responses [11,12], alleviating the suppressive effect of the hippocampus on HPA axis responses.

Although a causal relationship has not been conclusively established, there is abundant correlative evidence suggesting links between neurogenesis and depression, serotonin and adrenal glucocorticoid hormones (for review, see [4]). Chronic exposure to factors such as stress or corticosteroids that inhibit proliferation and survival of new cells have been shown to induce depression-related behavior in animal studies [13]. Stress and glucocorticoids play a major role in depression in humans [14–17], with a decrease in hippocampal size in human patients with major clinical depression [18], the magnitude of which is correlated with the length of depressive illness [19–21]. Neurogenesis appears to be required for the behavioral effects of antidepressants [22] on a time course corresponding to restoration of neurogenesis by these agents in mice [23], although this relationship is questionable in primates and humans in which the rates of neurogenesis are prolonged considerably beyond the time course for antidepressant effects [24]. Antidepressants are also thought to enhance cell survival in the dentate gyrus and have also been shown to reverse the inhibitory effects of stress on hippocampal cell proliferation in rodents [25].

The effects of stress on neurogenesis, which are mediated in part through elevated corticosteroids, are also well-documented. Chronic restraint stress can produce depression- and anxiety-related behaviors in rodents [13,26]. In rats, restraint stress produces a decrease in hippocampal cell proliferation, survival and differentiation that can be restored by enriched environment [10,27]. In rats, chronic mild unpredictable stress induces anhedonia [28] -- a core symptom of clinical depression in humans -- which can be reversed with antidepressant drugs [29].

ThioTEPA was selected for this and previous studies [30,31] in large part due to its ability to readily penetrate the blood-brain barrier [32]. ThioTEPA is effective in the treatment of various solid tumors but has not been widely used clinically given its toxic effects on marrow. However, there have been numerous studies proposing inclusion of high-dose thioTEPA in the treatment of malignancies with subsequent bone marrow transplants [33–37], particularly in pediatric patients who we presume would be especially susceptible to the effects of impaired neurogenesis.

As depression addresses a psychological state, rodent models are limited to the study of depression-related behaviors reflective of some of the major symptoms of clinical depression in humans. In a previous study employing the Porsolt forced swim test and the tail suspension test, our group found no indication of behavioral despair in mice at times following treatment with chemotherapy at which both hippocampal cell proliferation and impairments in learning and memory were documented [31].

In this study, we evaluated the effects of the chemotherapy drug, thioTEPA (TT), on anhedonia behavior in mice. Given the prevalence of psychological stress in chemotherapy patients, we also examined the effects of thioTEPA on depression-related behaviors and corticosterone responses in mice subjected to chronic stress.

2. Materials and Methods

2.1. General

Subjects were 40 male C57BL/6J mice received from Jackson Laboratories (Bar Harbor, ME) at 5 weeks of age. Mice were individually housed under 12:12 light–dark cycle, with food and water provided ad libitum. At 56–60 days of age, mice were randomly assigned to receive three daily injections of either thioTEPA (TT; 10 mg/kg, i.p.) or vehicle (phosphate buffered saline; PBS). This regimen of thioTEPA administration is comparable to that administered to human cancer patients [36,37], although it is difficult to establish how physiologically comparable this regimen is given potential differences in metabolism and clearance between mice and humans; however, this regimen has previously been shown to significantly diminish cell proliferation in the dentate gyrus with minimal observable health implications for the mice [30,31]. Mice were later subjected to behavioral testing and histology at 20 weeks of age (Fig. 1, and described below).

2.2 Sucralose preference test procedure

Anhedonia was measured via sucralose preference test conducted 12 weeks after chemotherapy treatment. The artificial sweetener sucralose was used to avoid potential caloric effects of sucrose. A preliminary concentration comparison in a separate group of mice experiment determined that 0.01% sucralose solution was most highly preferred by mice. For anhedonia experiments, mice were given two pre-weighted drinking bottles filled with water, weighed daily to determine the volume ingested by each mouse each day. After two days measuring water consumption, one bottle was refilled with sucralose solution for two additional days of measurements. Bottle positions were reversed each day to determine if mice had preferences based on bottle position or for particular bottles. This regimen was conducted just prior to beginning chronic stress treatments, and was repeated beginning 7 days and 14 days into stress treatments. Average 2-day preference indices for each mouse (volume of sucralose solution as a percentage of total volume ingested) were subjected to three-way ANOVA (drug, stress, and time factors) followed by post-hoc Tukey analysis.

2.3. Restraint stress procedure

Following the baseline sucralose preference test, half the mice in each drug treatment group were subjected to daily restraint stress by wrapping each mouse in a 12×12 cm piece of aluminum window screen folded and stapled together to restrict movement by the mouse, while allowing urine to escape and preventing heat buildup associated with solid restraint devices. Each restrained mouse was placed in its home cage for the duration of the restraint, while unrestrained mice were left untreated in their home cages. To minimize potential acclimation to the daily restraint regimen, mice were alternately restrained for 2, 4, or 8 hours a day, beginning at 8, 6, or 4 hours into the light period, respectively.

2.4. Corticosterone measurements

Serum corticosterone (CORT) measurements were made from 200 µl samples of blood taken via retro-orbital eye bleeds on the day prior to the beginning of restraint stress, and following release from the 2-hour daily restraint sessions on day 7 and on day 14 of the restraint regimen. Samples were allowed to coagulate for an hour then briefly centrifuged at low speed, and the plasma removed by pipet and stored at –20°C. Once all the samples were

collected, corticosterone content was assayed in duplicate by ELISA (AssayMax kit, AssayPro Co., St. Charles, MO). Average corticosterone values for each brain were subjected to three-way ANOVA (drug, stress and time factors) followed by post-hoc Tukey analysis when appropriate.

2.5. Forced Swim Test procedure

The day after completion of anhedonia measurements and blood sampling, mice were subjected to a Porsolt forced swim test as described previously [31]. Time spent floating (as opposed to displaying escape behaviors) by each mouse in 3-minute intervals of a 9-minute trial was measured by automated video tracking system (FST Forced Swim Test software, Biobserve Co., Germany). Animals were tested only once, after completion of all other measurements, as we considered swimming a confounding stressor for the mice. Data were subjected to two-way ANOVA (drug and stress factors) followed by Tukey analysis when appropriate.

2.6. Hippocampal cell proliferation quantification

In order to evaluate the effects of stress and/or chemotherapy on proliferation of cells in the dentate gyrus of the hippocampus, a random subset of mice ($n=4$) from each treatment group were injected with EdU (5-ethynyl-2'-deoxyuridine; 50 mg/kg i.p. in PBS) immediately following their forced swim test, and perfused transcardially 30 minutes later with heparinized saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). A shortened exposure to EdU was employed based on preliminary data from our previous study [31] which raised concerns that the stress of the behavioral tests (especially the forced swim test and the tail suspension test) would themselves impair cell proliferation in the time period immediately following the test, and so reduced the "incubation time" for EdU to 30 minutes after verifying that cell labeling was reliably consistent, albeit slightly lesser in number, than for a 2-hour incubation (personal observation, unpublished). Following overnight post-fixation at 4–8°C, brains were immersed in 30% sucrose in PBS for >24 hrs and sectioned at 30 μ m coronally throughout the rostro-caudal extent of the hippocampus using a cryostat (Bright OTF5000, Bright Instruments, UK). A series containing every 7th section throughout the hippocampus for each brain was mounted on Superfrost Plus coated slides and subjected to histochemical fluorescent detection of EdU (Click-It kit, Molecular Probes Inc., Eugene, OR), and EdU-labeled cells in the subgranular zone were quantified by an investigator blind to the treatment of each sample to generate an estimate of dentate cell proliferation in each brain. Histology for EdU-labeled cells [38,39] was used in light of superior staining in our experience (more intense specific staining on lower fluorescent background, using a quicker and easier procedure) after confirming that cell counts in the dentate gyrus of an independent sample of mice were comparable to traditional immunohistological staining for BrdU (unpublished data). Group data were subjected to two-way ANOVA (drug treatment and stress treatment factors) followed by post-hoc Tukey analysis when appropriate.

3. Results

3.1. Effects of thioTEPA treatment and restraint stress on sucralose preference

Prior to restraint stress, all mice consumed total average volumes of 4–5 ml of fluid each day, regardless of their prior drug treatment or whether they had sucralose or only water to drink. No significant or consistent preferences for side of cage or for a particular bottle were evident for any of the groups (ANOVA, $p>.05$). Sucralose preference was evident and similar in all mice prior to stress (Fig. 2A). Over the first week of the stress regimen, sucralose preference rose significantly for mice not receiving chemotherapy, regardless of whether they were subjected to restraint stress or not (Fig. 2B). After two weeks, both

stressed control mice and unstressed thioTEPA-treated mice had a diminished sucralose preference compared to control mice (Fig. 2C). Moreover, sucralose preference was completely abolished in thioTEPA-treated mice subjected to restraint stress. Over the course of the experiment, total volumes ingested by the mice when sucralose solution were available increased significantly from pre-stress levels (three-way ANOVA, $F(2,186)=3.534$, $p=.031$) for all groups that demonstrated sucralose preference.

3.2. Effects of thioTEPA treatment and restraint stress on corticosterone responses

Corticosterone (CORT) measurements in those same mice revealed slightly but significantly elevated levels in mice previously treated with thioTEPA (Fig. 3A). After one week, restraint stress yielded a significant elevation CORT levels regardless of prior chemotherapy treatment (Fig. 3B) that was exacerbated in thioTEPA-treated mice after two weeks (Fig. 3C).

3.3. Effects of thioTEPA treatment and restraint stress on forced swim test performance

In the forced swim test, thioTEPA-treated mice subjected to restraint stress spent significantly less time in escape behavior and more time floating during the first 3-minutes of the test than the other groups (two-way ANOVA, $F(1,31)=.847$, $p<.05$) (Fig 4). Similar differences were seen in the second and third 3-minute segments of the trial, though did not quite reach statistical significance.

3.4. Effects of thioTEPA treatment and restraint stress on hippocampal cell proliferation

Cell proliferation in the dentate gyrus of the hippocampus was significantly diminished in thioTEPA-treated mice (two-way ANOVA, $F(1,12)= 87.6$, $p<.05$) (Figs. 5 and 6), consistent with results published previously by our group [31]. Cell proliferation was also significantly inhibited by chronic restraint stress regardless of chemotherapy treatment. No interaction effect of stress and thioTEPA was observed ($p>.05$).

4. Discussion

Results of these experiments demonstrate that while prior thioTEPA treatment does not itself induce depression-related behaviors in mice, it does have the capacity to exacerbate depression-related behaviors as well as glucocorticoid secretion induced by psychological stress imposed several months subsequent to the chemotherapy treatment, suggesting that stress management may merit examination as a therapeutic intervention to reduce the prevalence or severity of depression in chemotherapy patients.

It is noted that the approximate 3-fold preference for sucralose solution observed in all mice during their baseline pre-stress measurements increased significantly in vehicle-treated but not thioTEPA-treated mice over the first week of the experiment. It may be that the mice experienced some neophobia during their first two-day baseline exposure to sucralose that was overcome in subsequent exposures. This is supported by the observation that total volumes ingested by the mice were increased for groups in which sucralose preferences were observed, suggesting that mice ingested greater volumes when they found sucralose preferable. It is unlikely that thioTEPA rendered mice unable to detect sucralose, as there was an initial preference for it in thioTEPA-treated mice comparable to that in vehicle-treated mice, which was only later diminished by daily exposure to restraint stress.

Whether elevated corticosterone contributed directly to diminished sucralose preference cannot be determined from this experiment. The modest baseline elevation of CORT in thioTEPA-treated mice (the exact duration of which is unknown) was not associated with anhedonia. Similarly, one-week exposure to restraint stress that yielded a considerable

increase in CORT did not induce anhedonia in control mice. However, anhedonia was observed in mice in which CORT was elevated for two weeks regardless of prior drug treatment, suggesting that the duration as well as the magnitude of CORT elevation may be related to any effects it may have on anhedonia.

Previous studies by Schloesser et al. [11] and Snyder et al. [12] suggest that hippocampal granule cells play a role in regulating HPA axis responses to stress, and that impairment of neurogenesis diminishes the ability to recover from psychological stressors, as evident in both elevated and prolonged CORT responses. Previous work from our lab [31] indicates that baseline cell proliferation in the dentate gyrus is significantly impaired for up to 20 weeks following thioTEPA treatment. In this study, a modest elevation of CORT was observed in unstressed mice treated 12 weeks earlier with thioTEPA, similar to results of Schloesser et al. in transgenic mice with chronically diminished neurogenesis [11], possibly reflecting a moderate disinhibition of stress responses typically regulated by the hippocampus. Moreover, following daily restraint stress, the modest disinhibition by thioTEPA yielded exacerbated stress-induced responses comparable to the effects observed in other studies in which neurogenesis was impaired by alternative means [11,12], suggesting a mechanism wherein dampened regulation of HPA axis resulting from prior thioTEPA treatment leads to elevated CORT secretion which could further inhibit neurogenesis, producing a feed-forward loop of heightened sensitivity to stress. Additional assessments of neurogenesis beyond measurement of cell proliferation would be required to substantiate such an underlying mechanism in our model.

Cell proliferation in the dentate gyrus was significantly suppressed by our regimen of daily restraint stress, which we speculate may have resulted from elevated corticosterone levels, previously shown by others to impair cell proliferation in the rodent dentate gyrus [7–9]. We predicted an additive effect of suppressed cell proliferation from prior thioTEPA treatment in the chronically stressed mice, an effect that was not evident in the data. We note that the variability in cell proliferation in this group is much smaller than in the groups that received only one or the other of those two treatments, and propose that any such interaction of elevated corticosterone and prior thioTEPA treatment may have had the effect of reducing two variable inhibitory effects to a floor level of proliferation below which it is difficult to suppress cell proliferation further, possibly as a result of progenitor regeneration from glial or dormant stem cell populations that has been shown to occur following insult in the dentate gyrus [40,41]. Alternatively, it is likely that the effects of thioTEPA and/or stress may be acting not only on proliferation but also on maturation, integration and/or survival of newborn neurons in the dentate gyrus, and that the additive effects of thioTEPA and stress were not evident in the measurements of cell proliferation made in this study.

We also note the inconsistency in results of the sucralose preference test (in which all treated groups demonstrated increased depression-related behavior relative to controls) and in the forced swim test (in which only animals experiencing both stress and thioTEPA treatment demonstrated exaggerated behavior relative to controls). This disparity may have resulted from differential sensitivities of the two tests, wherein more subtle intermediate effects were not detected in the forced swim test, which is itself a highly stressful experience for mice that encompasses other physiological challenges including physical exertion and thermal regulation. Alternatively, the disparity in results between the two tests may simply reflect the fact that they measure two different facets of a syndrome that may develop independently from one another, temporally or mechanistically.

Most notable of these results is the exaggerated corticosterone response resulting from chronic daily stress in thioTEPA-treated mice. It is not apparent from this experiment whether the magnitude or the duration of such effects would be maintained over even longer

periods of time, but adverse health effects of long-term corticosteroid exposure are well-documented and should be investigated further, given that putative dysregulation of corticosteroids may have effects detrimental to the recovery and remission from cancer even beyond those on depression-related behavior modeled in these experiments.

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Research Highlights

- Chemotherapy drug thioTEPA does not induce depression-related behaviors in mice.
- Prior thioTEPA treatment exacerbates stress-induced anhedonia in mice.
- Prior thioTEPA treatment with subsequent stress impairs forced swim test performance.
- Stress-induced corticosteroid responses are exacerbated by thioTEPA.
- No interaction of thioTEPA and stress on hippocampal cell proliferation was observed.

Fig. 1.

Schematic flowchart of experiment design. At 8 weeks of age, mice were subjected to 3 daily injections of thioTEPA or vehicle injections (TT/veh) and returned to group cages. Twelve weeks later, mice were separated into individual cages and subjected to a 2-day sucralose preference test (SPT), followed by a retro-orbital eyebleed to measure corticosterone (Cort). They were then subjected to 2–6 hours of daily restraint stress (R) for 14 days; controls were not manipulated. On the last two days of the restraint regimen, they were subjected to another sucralose preference test (SPT). Upon release from their restraint session on day 7 and day 14 of the regimen, blood samples were taken for corticosterone measurements (Cort). On the day following the restraint regimen, mice were subjected to a Porsolt forced swim test (FST), after which 4 mice from each group were immediately injected with Edu and perfused 30 minutes later for histology to assess cell proliferation in the dentate gyrus of the hippocampus.

Fig. 2.

Stress-induced anhedonia is exacerbated by prior thioTEPA treatment. (A) Moderate sucralose preference measured 12 weeks following chemotherapy was evident in control and thioTEPA-treated mice. (B) After one week, sucralose preference increased in control mice but not thioTEPA-treated mice, regardless of whether they were subjected to daily restraint stress. (C) After 2 weeks of daily restraint stress, sucralose preference was diminished in control mice, and was completely abolished in thioTEPA-treated mice. Data shown are mean $\pm$ SEM. * $p < .01$, three-way ANOVA followed by Tukey post-hoc comparison. *a, b* subscripts denote significant differences among all groups as revealed by post-hoc analysis. Dashed line indicates 50% (random) preference.

Fig. 3. Stress-induced corticosterone (CORT) responses are exacerbated by prior thioTEPA treatment. (A) Baseline CORT levels were slightly elevated in thioTEPA-treated mice. (B) One week of daily restraint stress produced significant elevations in CORT levels in both control and thioTEPA-treated mice. (C) After two weeks of daily restraint stress, stress-induced CORT responses were exaggerated in thioTEPA-treated mice. Data shown are mean $\pm$ SEM. * $p < .01$, three-way ANOVA followed by Tukey analysis. *a, b, c* subscripts indicate significant differences among all groups revealed by post-hoc analysis.

Fig. 4. Two weeks of daily restraint stress produced increased depression-related behavior in thioTEPA-treated mice. Data shown are time spent floating (as opposed to displaying escape-related behavior) in the first three minutes of a Porsolt forced swim test (mean $\pm$ SEM). * $p < .05$, two-way ANOVA followed by Tukey analysis.

Fig. 5. Representative histology for EdU in the dentate gyrus of this hippocampus. The number of labeled newborn cells in control mice (A) was significantly reduced in mice subjected to 2 weeks of daily restraint stress (B), thioTEPA treatment 12 weeks previously (C), or both treatments (D). Images were captured at 50X magnification. Insert in panel A is 400 $\times$ magnification.

Fig. 6. Cell proliferation in the dentate gyrus is impaired by prior thioTEPA treatment or chronic daily restraint stress. Data shown are estimates of total newborn cells from samples of EdU-labeled cells in every 7th section through the rostro-caudal extent of the dentate gyrus (mean $\pm$ SEM). The effect of combined thioTEPA and chronic stress was not significantly different than either treatment alone. * $p < .05$, two-way ANOVA followed by Tukey analysis.