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ORIGINAL INVESTIGATION

The impact of sustained and intermittent docetaxel chemotherapy regimens on cognition and neural morphology in healthy mice

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Abstract
Rationale A subset of cancer survivors demonstrates impairments in cognition long after chemotherapy completion. At present, it is unclear whether these changes are due to direct neurotoxic effects of chemotherapy.
Objectives This study examined the impact of variable docetaxel (DTX) chemotherapy dosing on brain DTX exposure via analyses of neural morphology and changes in cognition.
Methods Male CD-1 mice were treated with DTX either intermittently (8 mg/kg i.p. weekly) or via a sustained delivery system (DTX-PoLi$_{gel}$), which continuously releases DTX. Both groups received total DTX doses of 32 mg/kg. Mice were assessed on the novel object recognition (NOR) task and the Morris water maze (MWM) shortly after treatment.
Results Post-treatment behavioral testing demonstrated impaired NOR in mice treated with either dosing schedule relative to controls. No differences were observed between groups in MWM training and initial testing, though control mice performed better than chance while DTX-treated mice did not. Appreciable amounts of DTX were found in the brain after both treatment regimens. DTX treatment did not significantly increase levels of apoptosis within the CNS. However, some elevation in neural autophagy was observed following DTX treatment. Analysis of astrocytic activation demonstrated that intermittent DTX treatment resulted in an elevation of GFAP-positive astrocytes for 48 h after administration. Sustained chemotherapy demonstrated prolonged but lower levels of astrocyte activation over 9 days following implantation.
Conclusions DTX treatment induced cognitive impairment shortly after treatment. Further, these findings suggest an association between DTX dosing, neurotoxicity, and cognitive effects.

Keywords Chemobrain · Chemotherapy · Cognition · Memory · Docetaxel · Autophagy · Mice

Abbreviations

BBB	Blood–brain barrier
CNS	Central nervous system
DAPI	4′,6-Diamidino-2-phenylindole
DTX	Docetaxel
GFAP	Glial fibrillary acidic protein
HPLC–MS/MS	High-performance liquid chromatography tandem mass spectrometry
i.p.	Intraperitoneal
MWM	Morris water maze
NOR	Novel object recognition
P-gp	P-glycoprotein
PTX	Paclitaxel

Introduction

Advances in chemotherapy have contributed to a reduced risk of cancer recurrence and improved patient survival rates (Mamounas et al. 2005). However, a significant subset of

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cancer survivors treated with chemotherapy exhibit impairments in cognition with respect to verbal and visually linked memory tasks, as well as executive function and task processing speed following completion of treatment (Vardy and Tannock 2007). Furthermore, there is evidence to suggest that such deficits in cognition persist for years following treatment (Schagen et al. 2006; Silverman et al. 2007). Although a diagnosis of cancer appears to be associated with cognitive impairments prior to chemotherapy (Ahles et al. 2008), there is significant evidence to suggest that chemotherapy treatment may induce cognitive impairments. Specifically, such cognitive deficits are also observed in cancer-free rodents treated with drugs such as methotrexate (MTX), 5-fluorouracil (5FU), cyclophosphamide, and doxorubicin (Foley et al. 2008; MacLeod et al. 2007; Seigers and Fardell 2011; Seigers et al. 2008).

Docetaxel (DTX) is a taxane chemotherapeutic agent widely used in the treatment of breast, ovarian, non-small cell lung, head, and neck cancers (Clarke and Rivory 1999). While several studies on cognitive function in breast cancer patients have included women who received DTX-containing regimens (Quesnel et al. 2009; Reid-Arndt et al. 2009; Sadighi et al. 2006; Weis et al. 2009), these studies have not evaluated the ability of DTX-containing regimes to impair cognitive function with respect to other treatment regimes. Thus, the effects of systemic DTX treatment on cognition remain largely unknown.

With respect to the degree and persistence of relative cognitive impairment, it is possible that the dosage and schedule of chemotherapy may affect this outcome. Clinical studies suggest a link between higher doses of chemotherapy and cognitive impairment (Schagen et al. 2006). The impact of different schedules of chemotherapy administration is less clear. Preclinical studies in murine models of ovarian cancer have demonstrated that exposure to continuous low-dose DTX via a continuous release injectable formulation, DTX-PoLi$_{gel}$, is more effective and less toxic than standard intermittent regimens (De Souza et al. 2010). DTX-PoLi$_{gel}$ is an injectable, biodegradable hydrogel formulation which provides sustained release of DTX for over 28 days (Zahedi et al. 2009, 2011). However, it is unknown how this form of drug exposure might affect cognition. Furthermore, the mechanisms behind such impairments are currently unclear. Previous studies have suggested a role for decreased neurogenesis, increased cell death, and possible inflammatory processes (Seigers and Fardell 2011). Intermittent therapy incurs repeated cycles of high plasma concentrations often resulting in dose-limiting toxicities; thus, we hypothesized that continuous administration of DTX with DTX-PoLi$_{gel}$, resulting in substantially lower systemic concentrations, would cause less impairment of cognitive function than in mice administered similar total intermittent doses of DTX. Therefore, in the present study, we assessed the respective impact of systemic intermittent and sustained DTX administration on brain exposure to DTX, neural morphology, and cognition in healthy mice. We also examined the impact of treatment strategies on P-glycoprotein (P-gp) expression in the brain as DTX is a substrate of P-gp, a drug efflux transporter which can limit DTX accumulation into the brain (Fromm 2004).

Materials and methods

Materials

Anhydrous DTX (98 %) was purchased from Jari Pharma Ltd. (Lianyungang, China). Taxotere® (Aventis Pasteur), the commercial formulation of DTX, was obtained from Mount Sinai Hospital Pharmacy (Toronto, Canada). Paclitaxel (PTX) was purchased from PolyMed Therapeutics (Houston, TX, USA) and used as the internal standard for quantitative high-performance liquid chromatography tandem–mass spectrometry (HPLC–MS/MS) analysis. Egg phosphatidylcholine (ePC) and C12 lauric aldehyde were purchased from Sigma-Aldrich Chemical Co. (Oakville, Canada) and used without further purification. The water-soluble chitosan derivative and DTX-PoLi$_{gel}$ were prepared as described earlier (Cho et al. 2006; Zahedi et al. 2009). Briefly, DTX was dissolved in ethanol, dried under nitrogen, and resuspended in a solution of ePC, lauric aldehyde, and water-soluble chitosan to achieve a final DTX/chitosan–phospholipid blend of 1:8 (w/w) and then sterilized under UV light. USP-grade isoflurane was purchased from Baxter Corporation (Mississauga, Ontario, Canada) to anesthetize the experimental animals.

DTX treatment

Male CD-1 mice (Charles River) weighing between 27 and 33 g (mean, 30.1±1.6 g) were used for all studies. Mice were housed in groups of three or four and maintained at 21 °C on a 14:10-h day/night light cycle. All procedures were approved by the University of Toronto Animal Care Committee and the Canadian Animal Care Council. Mice in the sustained treatment group were given a single intraperitoneal (i.p.) injection of an injectable continuous DTX delivery system, DTX-PoLi$_{gel}$, which continuously releases 8 mg/kg/week for a total dose of 32 mg/kg over 4 weeks (De Souza et al. 2010). Mice in the intermittent treatment group received four i.p. injections of Taxotere® (8 mg/kg) on days 0, 9, 18, and 28 for a total DTX dose of 32 mg/kg. This dose has been shown to significantly reduce tumor growth in murine xenograft models (DeSouza et al. 2010). Mice in the control group received saline injections (matched for volume) on the same days as the

intermittent group to account for physical handling and injections.

Behavioral testing: novel object recognition

Twenty-four hours after the last DTX treatment in a dimly lit room, mice (n =9–12/group) were habituated to the opaque novel object testing arena (measuring 40 cm wide×30 cm long) for 10 min in the absence of any objects. The following day, mice were tested for object recognition using a paired-sample test trial separated by an 80-min delay. This delay was chosen based on earlier pilot testing conducted in the laboratory and found to be sensitive to DTX-induced effects on cognition. During the sample trial, mice were exposed to two identical objects (either a pair of solid textured half-sphere glass or a pair of plastic half cylinders) for 10 min. During the test trial, mice were exposed to the original object and a new, "novel" object (either an upturned match box car or glass jar). All objects were counterbalanced between the groups, and the objects and arena were cleaned with 50 % ethanol between trials to eliminate odor cues. The dependent variables of interest were the amount of exploration time on each object and preference for the novel object, calculated as the amount of time spent investigating the novel object as a percent of the total time spent investigating both the novel and familiar objects (Fardell et al. 2010). Object investigation was defined as previously described (Ennaceur and Delacour 1988); mice were considered to be investigating the object when their nose was directed at the object and within 0.5 cm of the object. Biting or sitting on the object was not considered investigation. All trials were scored by an observer blind to the treatment group.

Behavioral testing: Morris water maze

All training and testing took place in a white, opaque maze (122 cm diameter×76 cm height) and employed a procedure similar to that previously described, with the absence of a fear cue (Bonsignore et al. 2008). The water was made opaque by the addition of nontoxic water-soluble paint and maintained at 30±1 °C. Training commenced 7 days following completion of DTX treatment, with mice receiving four trials per day for two consecutive days. During training, the platform (15-cm diameter) was kept hidden from the mouse's view and submerged 1 cm below the surface of the water. The platform remained in the same location, the NE quadrant, throughout the training. The start location of the trials was random and differed from trial to trial. Training trials lasted 1 min or until the mouse found and climbed onto the hidden platform, whichever came first. Once the mouse was on the platform, it was allowed to remain there for 20 s. Mice were dried and returned to their home cage during the intertrial interval for approximately 30 min. Test trials were conducted 24 h after the last training trial (9 days post-treatment) and again 7 days later (16 days post-treatment). A timeline of behavioral testing is provided in Fig. 1. During testing, the platform was not present and mice swam freely for 1 min. In this time, the latency to cross the platform location, time spent in the target quadrant, distance from the target location, and number of errors (incorrect quadrant entries) were tracked using ANY-maze software (version 4.5, Stoelting Co., Wood Dale, IL, USA). Upon testing completion, mice completed a cued platform test consisting of two training trials to check for any differences in visual ability. During this test, the platform was placed in a novel location (NW) and hidden from view; a large black hexagonal prism mounted above the platform indicated the platform location. Mice were timed how long it took them to find and climb onto the platform with all four paws, before being removed from the pool.

Drug content analysis by HPLC–MS/MS

Mice were sacrificed at 1 h, 6 h, 1 day, 2 days, 9 days, and 29 days post-treatment initiation by cardiac puncture under isoflurane anesthetic, and plasma and brains were collected (n =4/group). For the intermittent and control groups, the sacrifice on day 9 occurred before any injection of DTX or saline. Brain samples were weighed and mixed with Milli-Q water (1:4, w/v) and homogenized (Polytron® PT2100, Kinematica AG, Switzerland). Five milliliters of *tert*-butyl methyl ether and internal standard (PTX) was added to 0.2 mL of plasma or brain homogenate, vortexed for 5 min, and centrifuged (AllegraTM 6R, Beckman CoulterTM, USA) at 3, 750 rpm for 20 min. The organic layer was transferred into a clean tube, dried under gentle nitrogen flow, and resuspended in 200 μL methanol/water (50:50, v/v). Liquid chromatographic separation was carried out on an Agilent 1100 liquid chromatography (LC) (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump and a mobile phase degasser. Ten microliters of sample was injected through a CTC/PAL autosampler and delivered by gradient mobile phase elution, which started with methanol and 10 mM of ammonium hydroxide (NH_4OH) at a ratio of 65:35 (v/v), followed by gradient increase in methanol to 100 % in 1 min, which was maintained for 2 min then equilibrated with 35 % NH_4OH for 4.5 min before the next injection. Flow rate was kept at 0.2 mL/min. A Zorbax Eclips Plus C18 column (4.6×50 mm; particle size, 5 μm; Agilent Technologies) was employed for the LC separation. The eluate from LC was directed into an API-4000 triple quadrupole mass spectrometer equipped with a turbo spray ion source (PE Sciex, Toronto, Canada). The quadrupoles were operated in the positive ion mode. The resulting multiple-reaction monitoring chromatograms were used for quantification through Analyst software version 1.2 (PE Sciex). Mass transitions of 830.3>549.3 m/z

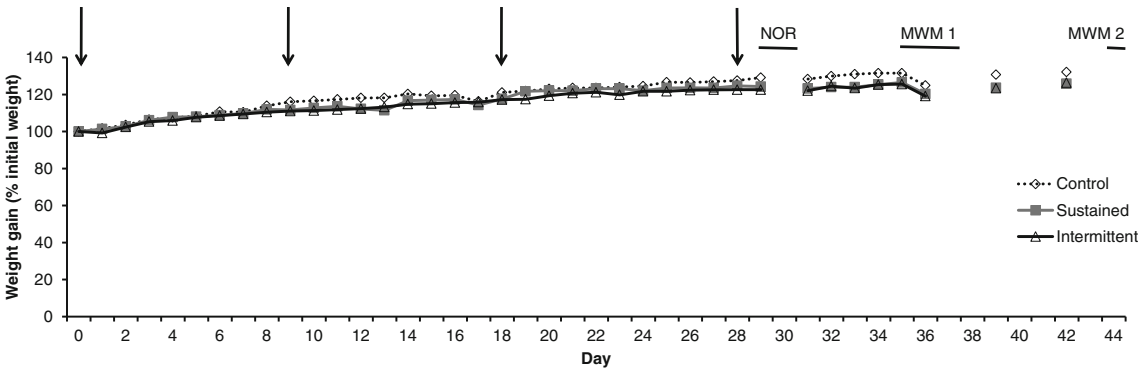

Fig. 1 Effect of DTX treatments on weight gain. Weight of mice throughout the study period is expressed as a percentage of the initial weight. *Arrows* indicate days of injection. Control mice were treated with saline only; intermittent treatment consisted of one i.p. bolus injection of Taxotere on days 0, 9, 18, and 28; and sustained treatment consisted of one i.p. $PoLi_{gel}$ injection on day 0 (total DTX dose for both groups, 32 mg/kg). Novel object recognition (*NOR*) days 29–30, Morris water maze training and test 1 (*MWM 1*) days 35–37, Morris water maze test 2 (*MWM 2*) day 44

and 876.3>308.1 *m*/*z* were optimized for DTX and PTX, respectively, with a dwell time of 200 ms. The curtain gas and collision gas (both N_2) were operated at 12 psi, and the ion spray voltage was kept at 5.5 kV, with a source temperature of 700 °C. The extraction efficiency of DTX from plasma and the brain was greater than 80 and 75 %, respectively. The limit of detection for DTX was determined as 0.1 ng/mL and 0.4 ng/g for plasma and brain samples, respectively. A calibration curve with a range of 0.3–1,000.0 ng/mL was made with eight-point calibrators using ratios of DTX to the internal standard (PTX) peak area.

Western blot analysis

Brains were harvested from control and treated mice on days 1, 2, 9, and 29 following initiation of DTX treatment or saline as described above (n=4/group). In each case, the entire brain was carefully removed, snap frozen in liquid nitrogen, and stored at −80 °C. Total protein extraction and quantification followed a previously established procedure (De Souza et al. 2011). Protein samples (40 μg) were separated by gel electrophoresis, transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA), and blocked with 5 % skim milk in Tris-buffered saline. Membranes were then incubated with primary antibodies against cleaved caspase-3 (1:500; Asp 175; Cell Signaling Tech) for indication of level of apoptosis, LC3 (1:500; LC3-B 98322; Abcam Inc. Cambridge, MA, USA) for level of autophagy, glial fibrillary acidic protein (GFAP) (1:10,000; Z0334; DakoCytomation, Denmark) for level of astrocytic activity, or P-gp (1:1,000; ab3364; Abcam Inc. Cambridge, MA, USA) overnight at 4 °C followed by incubation with anti-rabbit horseradish–peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were visualized using ECL Advance Western Blotting Detection kit (GE Healthcare, Buckinghamshire, UK) and FluorChem imager (AlphaEaseFC 6.0.014) and quantified using Alpha Ease FC imaging software (Alpha Innotch). Results were normalized to β-actin (1:10,000; AC-15; Sigma-Aldrich). The ratio of immunodetectable LC3-II to LC3-I was used to evaluate autophagy.

Immunohistochemistry

In order to determine spatial localization of neurochemical changes, we anesthetized a separate group of treated and control mice at 24 and 48 h after initiation of treatment (n=4/group). Mice were perfused via the left cardiac ventricle with a fixative solution of 4 % paraformaldehyde in 0.1 M phosphate buffered saline at pH 7.4. Fourteen-micron-thick sagittal sections were then obtained using a cryostat and stored at −20 °C. Representative sections from the hippocampus were collected on glass slides. Sections were quenched in methanol containing 3 % hydrogen peroxide for 30 min, washed in Milli-Q water, and incubated for 1 h in blocking buffer (0.3 % Tween 20, 5 % goat serum in phosphate buffered saline (PBS)). This was followed by overnight incubation at 4 °C with antisera to caspase-3 recognizing the cleaved isoform (1:200; Cell Signaling Technology), LC-3 (1:500; Invitrogen, Eugene, OR, USA), or GFAP (1:2,000; DakoCytomation, Denmark) in blocking solution. Sections were then washed for 5 min three times in blocking buffer. Secondary antisera (FITC-labelled anti-rabbit, 1:200 in blocking solution; Vector Laboratories, Burlingame, CA, USA) were added to sections and incubated for 1 h at room temperature. Stained slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) by incubating 300 nM DAPI staining solution (Invitrogen, Eugene, OR, USA) for 5 min then triple rinsed with PBS. All images were taken by fluorescent microscopy.

Statistical analysis

Results are expressed as mean±SE. Morris water maze (MWM) training trials were assessed with a two-way analysis of variance (ANOVA) with trial as the repeated measure and treatment group as the between-subjects measure. The average escape latency of the two cued platform trials was evaluated with a one-way ANOVA with the treatment group as the between-subjects factor. For all other data, one-way ANOVA was used, with treatment group as the between-subjects factor. Post hoc pairwise comparisons were performed using the Bonferroni correction procedure to control type 1 error rate. Findings were deemed significant if $p<0.05$. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, version 16.0).

Results

Impact of DTX treatment on weight and NOR performance

Figure 1 presents body weight gain as a percentage of the starting body weight prior to any experimentation. There was no main effect of treatment group ($F(2,30)=2.717$, $p=0.082$); however, there was a modest but significant day-by-treatment group interaction ($F(74,1110)=2.325$, $p<0.001$). On the day prior to initiating behavioral testing, there was no significant difference in weight between the treatment groups (mean control, 39.8 g (±0.7); intermittent, 38.1 g (±0.8); sustained, 37.8 g (±0.5); $p>.05$). During the novel object recognition (NOR) sample trial, there was no difference between the treatment groups in terms of total object exploration (mean control, 78.9 s (±2.8); intermittent, 95.8 s (±8.7); sustained, 82.5 s (±3.1); $p>.05$). During the test trial, a significant difference was observed between treatment groups with respect to level of exploration and novel object preference ($F(2,30)=4.008$, $p<.05$; $F(2,30)=22.219$, $p<.01$, respectively). Post hoc pairwise comparisons on test trial data found that intermittently treated mice showed reduced exploration compared to controls (mean control, 58.2 s (±4.1); intermittent, 39.7 s (±3.5); $p<0.05$), while mice receiving sustained DTX treatment did not differ significantly with respect to the controls (46.1 s (±5.7), $p>0.05$). Control mice showed greater novel object preference than either intermittent or sustained DTX-treated mice (Fig. 2; mean (±SE): control, 75.3 % (±1.7); intermittent, 53.3 % (±3.4); sustained 54.3 % (±3.0); $p<.001$).

Impact of DTX on MWM performance

During MWM training, there was a significant effect of training trial ($F(7,210)=10.473$, $p<0.001$), but no effect of group ($F<1$); all mice improved performance regardless of group. For test 1, no significant differences were found between the treatment groups on any of the variables of interest (Fs<1).

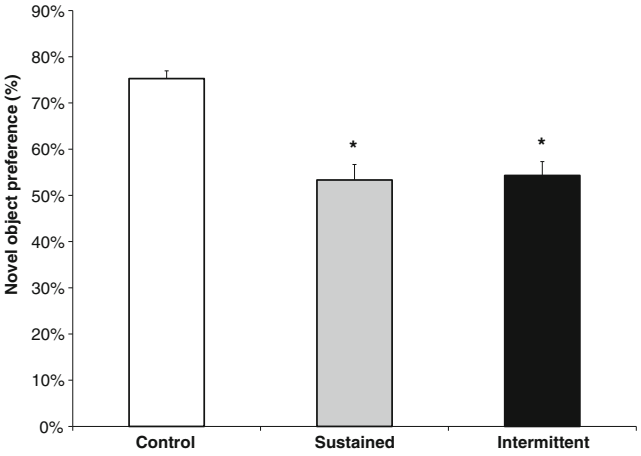

Fig. 2 Effect of DTX treatments on NOR performance. Novel object preference during the NOR test trial was calculated as the amount of time spent investigating a novel object as a percentage of the total time spent investigating both the novel and familiar objects. Control mice were treated with saline only, sustained treatment consisted of one i.p. $PoLi_{gel}$ injection on day 0, and intermittent treatment consisted of one i.p. bolus injection of Taxotere on days 0, 9, 18, and 28 (total DTX dose for both groups, 32 mg/kg). Data represent mean±SE ($n=9$–12/group). *$p<0.05$ (significant difference from controls). No differences were detected between treatment groups

However, control mice spent significantly more time in the target quadrant when compared to chance levels (mean, 33.2 % (±2.7); $t(11)=3.058$; $p=0.011$). In contrast, the two DTX treatment groups did not spend significantly more time in the target quadrant than chance (mean intermittent 25.9 % (±3.7), $p>0.05$; mean sustained 30.8 % (±3.4), $p>0.05$; see Fig. 3). In test 2, regardless of treatment group, mice did not spend significantly more time in the target quadrant than would be expected if they were performing at chance levels (mean control 25.1 % (±2.6), $p>0.05$; mean intermittent 25.4 % (±2.5), $p>0.05$; mean sustained 22.8 % (±3.2), $p>0.05$, Fig. 3). However, unlike test 1, there was a significant main effect of treatment group on the time taken to first enter the target quadrant ($F(2,32)=3.560$, $p<0.05$) and the distance traveled before the first entry to the target quadrant ($F(2,32)=3.540$, $p<0.05$). Post hoc comparisons found that control mice took less time to enter the target quadrant than mice treated with intermittent DTX ($p<0.05$); however, there was no difference between control and sustained DTX (mean (±SE): control, 5.16 s (±0.62); intermittent, 13.12 s (±2.42); sustained, 9.37 s (±2.70)). Similarly, intermittently treated mice swam a greater distance before entering the target quadrant than control mice ($p<0.05$) (mean (±SE): control, 1.32 (±0.22); intermittent, 3.34 (±0.63); sustained, 2.36 (±0.57)). There were no other significant differences during testing or cued platform testing (data not shown).

DTX plasma and brain concentrations following treatment

An initial burst of DTX was observed in plasma within the first 6 h of DTX-$PoLi_{gel}$ administration (Table 1), followed by

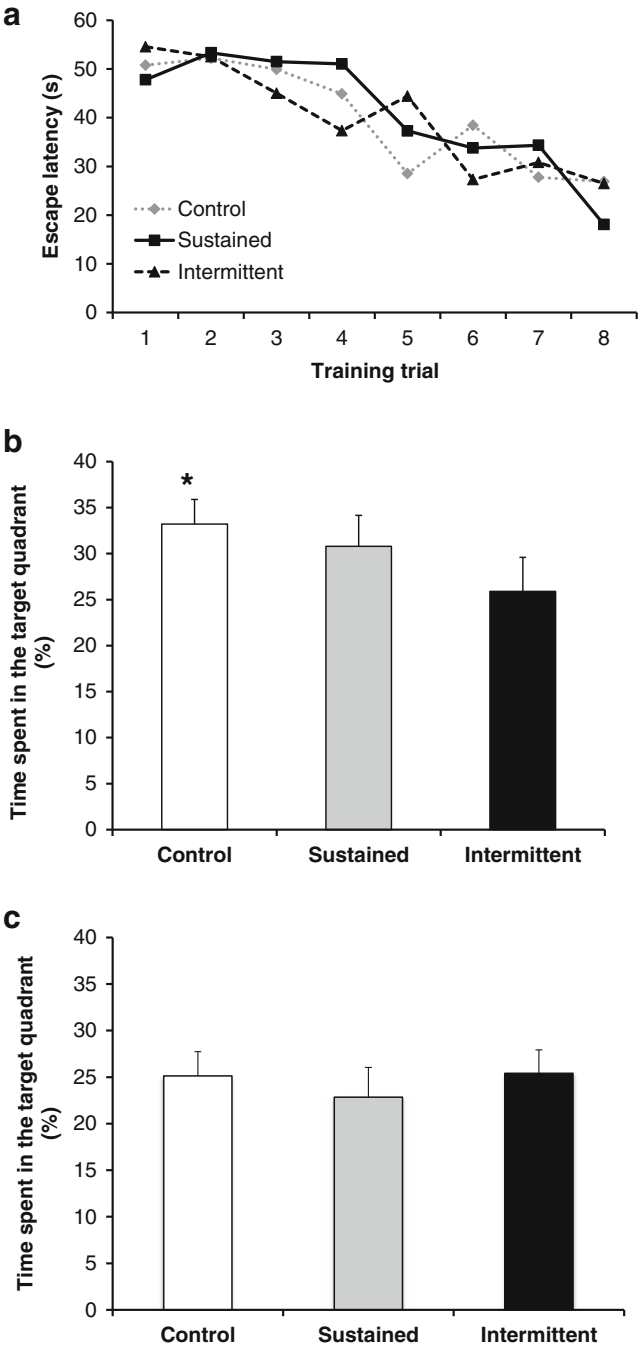

Fig. 3 Effect of DTX treatments on Morris Water Maze (MWM) performance. **a** Escape latencies during training trials. **b** Time spent in the target quadrant (%) during MWM probe test 1, immediately after training. **c** Time spent in the target quadrant (%) during MWM probe test 2, 7 days after the last test. Data represent mean time±SE (n = 9–12/group). Control mice were treated with saline only, sustained treatment consisted of one i.p. $PoLi_{gel}$ injection on day 0, and intermittent treatment consisted of one i.p. bolus injection of Taxotere on days 0, 9, 18, and 28 (total DTX dose for both groups, 32 mg/kg). *p < 0.05 (significant difference from chance level). No differences were detected between treatment groups

sustained DTX plasma levels until day 29 (range, 16.9–31.2 ng/mL). In the intermittent treatment group, DTX plasma

Table 1 DTX concentrations in the plasma (ng/mL) and brain (ng/g) of mice at various time points post-treatment with sustained or intermittent DTX as measured by HPLC-MS/MS analysis

	Sustained		Intermittent	
	Plasma	Brain	Plasma	Brain
1 h	60.3±42.1	0.4±0.4	1,228±96.9	28.3±3.2
6 h	92.0±15.8	3.2±1.8	26.8±24.1	9.5±4.5
24 h	16.9±3.6	1.5±0.9	1.9±1.3	1.4±1.0
48 h	23.3±5.7	7.6±2.8	1.6 ±1.2	0.0±0.0
Day 9	31.2±6.2	9.6±2.8	0.0±0.0	0.0±0.0
Day 29	23.1±9.4	0.0±0.0	24.2±3.0	10.0±2.4

Sustained treatment consisted of one i.p. $PoLi_{gel}$ injection on day 0, and intermittent treatment consisted of i.p. bolus injections of Taxotere on days 0, 9, 18, and 28. For the intermittent group, day 9 measurements of DTX were taken prior to injection of the second dose, while day 29 measurements were taken 24 h after the day 28 dose. Data represent mean DTX concentrations±SE(n = 4/group)

concentrations of 1,228±96.9 ng/mL were seen 1 h following injection, with levels decreasing to 26.8±24.1 ng/mL by 6 h after treatment. A further decrease to 1.6±1.2 ng/mL was observed 48 h following injection. On day 9, prior to the administration of the second dose in the intermittent group, DTX concentrations had fallen below detectable levels (<0.1 ng/mL). On day 29, 24 h following the last scheduled intermittent injection, DTX plasma concentration averaged 24.2± 3.0 ng/mL.

In the intermittent treatment group, DTX brain concentrations paralleled those observed in the plasma, with peak concentrations of 28.3±3.2 ng/g detected 1 h after administration followed by a rapid decline to 1.4±1.0 ng/g DTX within 24 h. Brain DTX levels were below the limit of detection 48 h post-injection and were similarly undetectable on day 9 prior to the next injection. On day 29, 24 h after the last intermittent injection, the mean brain DTX concentration was observed to be 10.0±2.4 ng/g. For the sustained DTX treatment group, we observed a general trend of increased accumulation of DTX in the brain (from 0.4±0.4 ng/g to 9.6±2.8 ng/g) from 1 h to 9 days post-administration; however, drug levels in the brain were not detectable on day 29.

Effect of DTX treatment on brain P-gp levels

Western blot analyses demonstrated a significant increase (~350 %) in P-gp expression 24 and 48 h after the first intermittent dose of DTX (Fig. 4) but returned to basal levels by day 9 prior to the second dose of DTX, indicating transient induction. Likewise, P-gp levels were significantly increased (~300 %) 24 h after the final intermittent dose on day 29. For the sustained treatment group, significant increases of 150–270 % in P-gp levels in the brain were detected on days 2, 9,

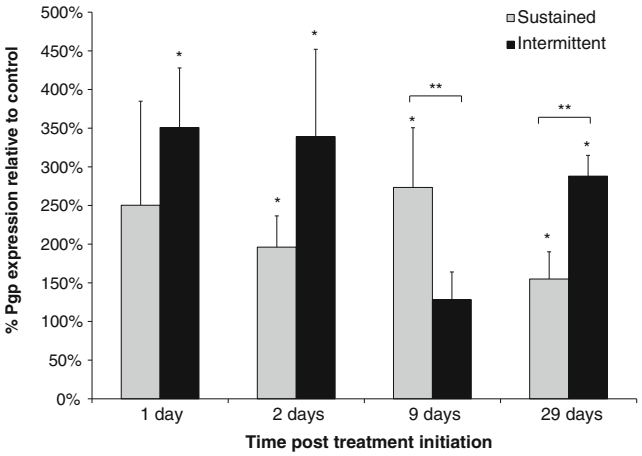

Fig. 4 Effect of DTX treatments on P-glycoprotein expression. P-gp protein expression in the brain relative to controls following sustained and intermittent DTX treatment, as measured by Western blotting. Sustained treatment consisted of one i.p. $PoLi_{gel}$ injection on day 0, and intermittent treatment consisted of one i.p. bolus injection of Taxotere on days 0, 9, 18, and 28 (total DTX dose for both groups, 32 mg/kg). Protein concentrations were normalized to β-actin. Data represent mean normalized P-gp protein concentrations±SD as percentages of control values (n=4/group). *p<0.05 (significant difference from controls), **p<0.05 (significant difference between treatment groups)

and 29 post-DTX-$PoLi_{gel}$ injection. Thus, sustained DTX exposure was associated with a prolonged induction of P-gp.

Effect of DTX treatment on brain apoptosis

To explore whether DTX acutely induces apoptosis in the brain, the pattern of activated caspase-3 was examined in both sustained and intermittent treatment groups using immunohistochemistry and Western blot analyses. Compared to controls, there was no significant increase in the levels of activated caspase-3 in either DTX treatment groups at 24 and 48 h post-treatment (data not shown).

Effect of DTX treatment on brain autophagy

Levels of activated LC3 (microtubule-associated protein 1A/1B-light chain 3) were measured as a ratio of LC3-II to LC3-I expression to assess the presence of autophagy. The degree of conjugation of the cytosolic form of LC3 (LC3-I) to phosphatidylethanolamine (LC3-II) is a general marker used to assess levels of autophagic activity. In the sustained treatment group, LC3-II formation did not significantly differ from that observed in controls (Fig. 5). However, within the intermittent treatment group, a significantly greater formation of LC3-II was observed 24 h following initial dosing. This was followed by a rapid decrease to control values. Likewise, on day 29, 24 h following the administration of the final dose, a significant increase in LC3-II formation was once again observed. Immunohistochemical analysis also detected increased LC3-II staining in the hippocampal sections of all DTX-treated mice at 24 h post-administration (Fig. 5b).

Effect of DTX treatment on astrocyte activation

Astrocytes exhibit a well-established cellular response to a range of neurologic insults. To assess the relative level of astrocytic activation in DTX-treated animals, GFAP was utilized as a marker of this response. Compared to controls, GFAP levels were elevated by approximately twofold on days 1, 2, and 9 (Fig. 6) in the sustained treatment group. In mice given intermittent doses of DTX, GFAP levels demonstrated a three- to fourfold elevation on days 1, 2, and 29 as compared to controls. Significant differences between treatment groups were also seen on days 9 and 29. Immunohistochemical analysis also detected increased GFAP staining in the hippocampal sections of all DTX-treated mice at 24 and 48 h post-administration (Fig. 6b).

Discussion

Clinical data have shown an association between chemotherapy treatment and long-term cognitive impairment in cancer survivors (Vardy and Tannock 2007). However, these clinical studies have been unable to clearly evaluate the impact of chemotherapy on cognitive function in the absence of cancer and other treatment-related drugs (e.g., antiemetics). In this study, we have evaluated the effect of the widely used chemotherapeutic agent DTX in the absence of malignancy and other potential confounders. Using different dosing schedules, we assessed brain drug exposure, degree of central nervous system toxicity as indicated by apoptosis, autophagy, astrocyte activation, and cognitive function in healthy mice.

This study demonstrated that both sustained and intermittent DTX treatment strategies were associated with subtle changes in behavior and cognition. Both the NOR and MWM are reliant on temporal lobe function. Tests of object recognition appear to rely on the perihinal cortex, while tests of location recognition and spatial reference memory are dependent on the hippocampus (D'Hooge and De Deyn 2001; Winters et al. 2008). There are functional distinctions in that spatial reference memories acquired during initial MWM training can be retained for days as opposed to hours as in the NOR (Ennaceur and Delacour 1988). DTX treatment induced clear deficits in object recognition and had subtle effects on spatial reference memory. Specifically, intermittent and sustained DTX-treated mice were equally impaired relative to the controls shortly after treatment completion and did not display a clear preference for the novel object. For the MWM, all mice demonstrated some spatial learning during training, but during probe test 1, which was run shortly after training, there was no main effect of treatment group on any of

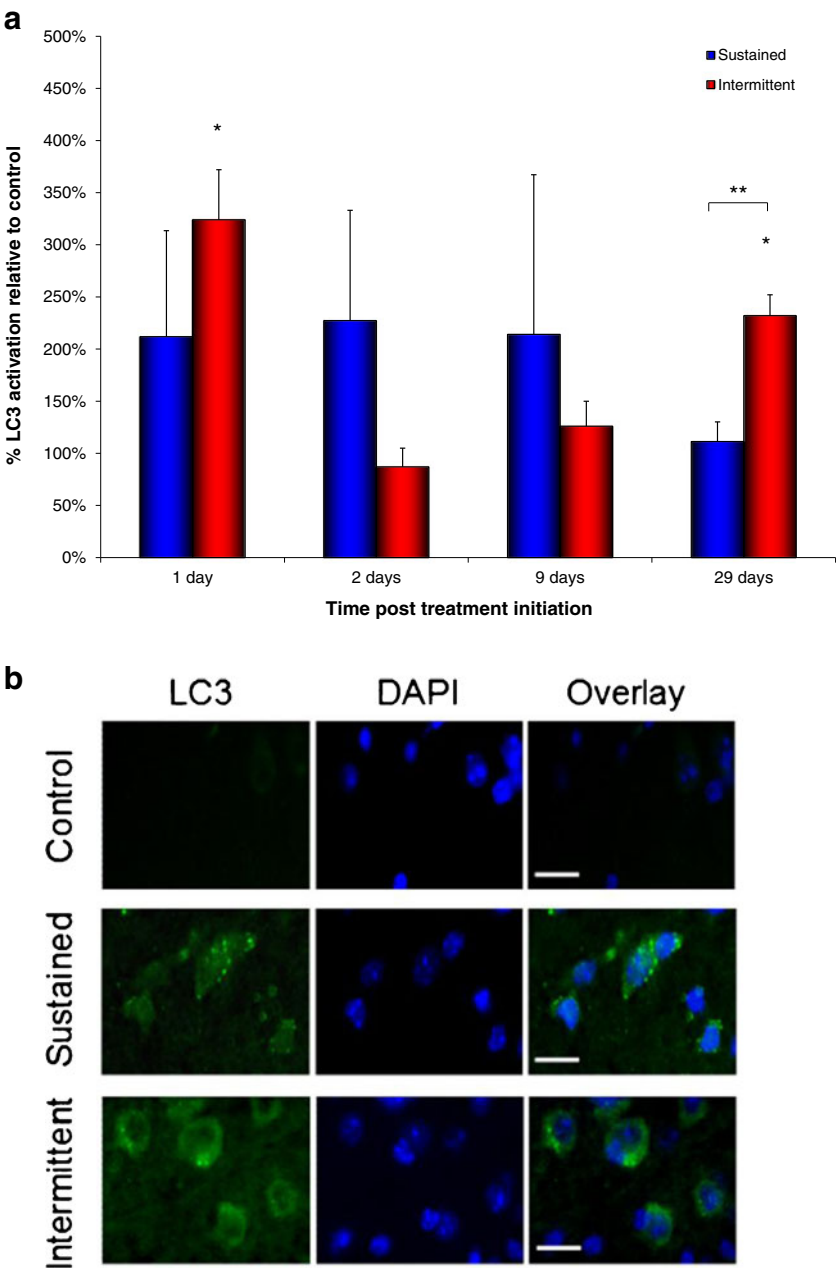

Fig. 5 Effect of DTX treatments on autophagy. **a** Relative quantification of brain LC3 protein expression following sustained and intermittent DTX treatment, as measured by Western blotting. Sustained treatment consisted of one i.p. $PoLi_{gel}$ injection on day 0, and intermittent treatment consisted of one i.p. bolus injection of Taxotere on days 0, 9, 18, and 28 (total DTX dose for both groups, 32 mg/kg). The ratio of LC3-II to LC3-I was used as a measure of LC3 activation, such that LC3-II protein concentrations were normalized to those of LC3-I. Data represent mean normalized LC3 protein concentrations±SD as percentages of control values ($n=4$/group). *$p<0.05$ (significant difference from controls), **$p<0.05$ (significant difference between treatment groups). **b** Representative images of brain sections immunostained with anti-LC3 antibody and counterstained with DAPI 1 day after intermittent and sustained treatment initiation. *Scale bars*, 0.25 μm

the variables of interest. However, mice treated with either intermittent or sustained DTX did not perform better than chance, while mice in the control did, and spent significantly more time in the target quadrant relative to the other quadrants during test 1. Interestingly, during probe test 2, which was run 7 days later, mice treated with intermittent DTX appeared to perform worse than controls, while sustained DTX-treated mice performed no different than the controls. This latter result is difficult to interpret. All mice, regardless of treatment group, did not spend significantly more time in the target quadrant than would be expected if performance was at chance level during the second probe test. As such, the poor performance of the intermittent DTX mice relative to controls may not necessarily reflect true lasting cognitive deficits. Regardless, taken together, the results presented here suggest that DTX treatment impacts performance in tests of cognition reliant on medial temporal lobe function shortly after treatment (D'Hooge and De Deyn 2001; Winters et al. 2008).

Treatment with DTX is associated with toxicities such as peripheral neuropathy in both rats (Otova et al. 2006; Persohn et al. 2005) and humans (Park et al. 2008). Although not measured here, it is possible that the mice treated with DTX would similarly experience symptoms such as mechanical or thermal allodynia that could impact performance in measures of cognition, particularly in the MWM. Importantly, no differences in swimming variables (speed, total distance) due to

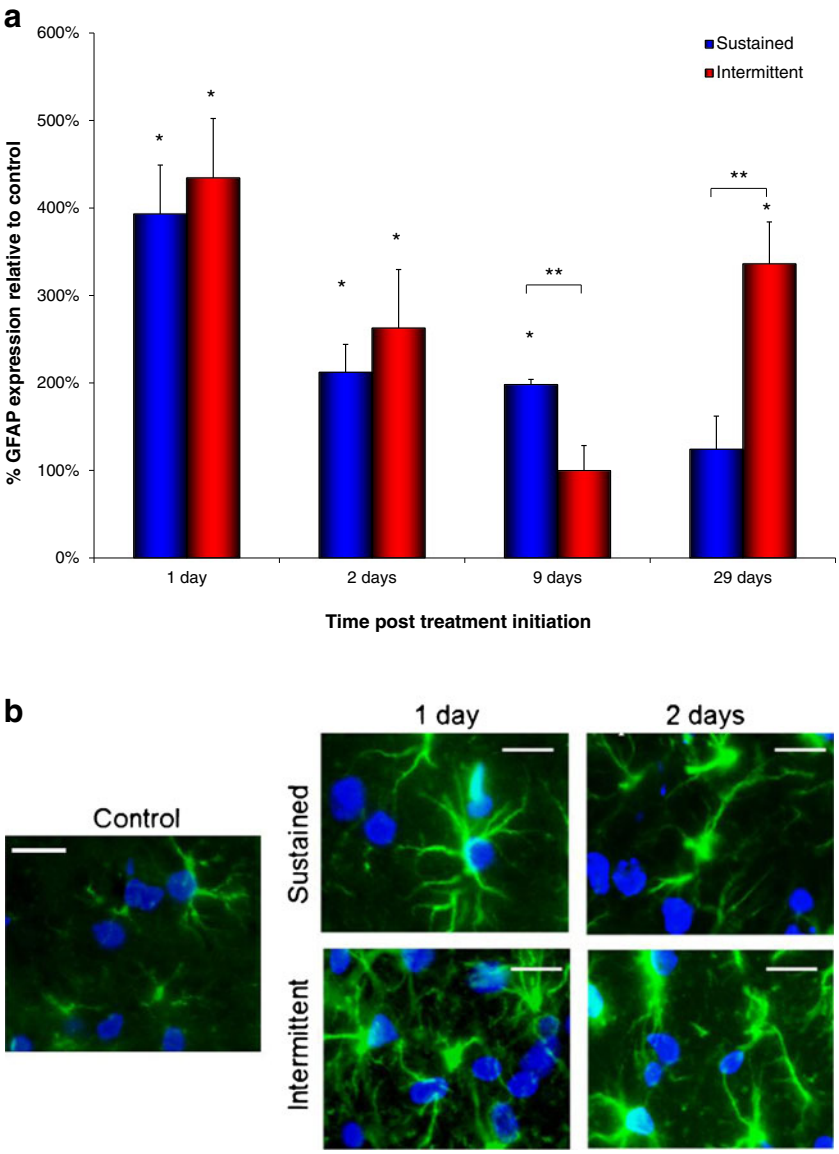

Fig. 6 Effect of DTX treatments on astrocyte activity. **a** Relative quantification of GFAP as a measure of brain astrocyte activity following sustained and intermittent DTX treatment, as measured by Western blotting. Sustained treatment consisted of one i.p. $PoLi_{gel}$ injection on day 0, and intermittent treatment consisted of one i.p. bolus injection of Taxotere on days 0, 9, 18, and 28 (total DTX dose for both groups, 32 mg/kg). Protein concentrations were normalized to β-actin. Data represent mean normalized GFAP protein concentrations±SD as percentages of control values (n=4/group). *p<0.05 (significant difference from controls), **p<0.05 (significant difference between treatment groups). **b** Representative images of brain sections immunostained with anti-GFAP antibody to identify activated astrocytes (*green*), and nuclei were counterstained with DAPI (*blue*) 1 and 2 days after initiation of intermittent and sustained treatment. *Scale bars*, 0.25 μm

DTX were observed in the present study; however, mice treated with intermittent DTX had lower levels of object exploration during the NOR test trial than the control. Although there was no difference in the level of object exploration during the NOR sample trial nor between control and sustained DTX-treated mice in the test trial, these results are suggestive of a differential impact of intermittent DTX on levels of motivation, anxiety, and/or motor output and may potentially reflect greater toxicity associated with intermittent DTX treatment. Analysis of weight data throughout the experimentation suggested that DTX treatment is associated with slight reductions in weight gain, but significant differences between treatment groups were not seen at the initiation of behavioral studies. It is unclear if these changes could impact performance in measures of cognition.

DTX is a semisynthetic analogue of PTX, and in contrast to the obtained results, Boyette-Davis and Fuchs (2009) found that PTX-treated rats displayed normal working memory, information processing speed, and visual attention as measured by the five-choice serial reaction time task (5CSRT), despite evidence of peripheral neuropathy. Several procedural differences may account for these contrasting findings. Although we cannot exclude the possibility that PTX may not induce changes in cognitive function, the dose employed by Boyette-Davis and Fuchs (2009) was low (1 mg/kg) and administered over a short time period of 12 days relative to the procedure employed here. In addition, rats were trained to complete the 5CSRT to criterion prior to treatment, while mice in our present study completed the cognitive tasks after all treatments were complete. Finally, the cognitive tasks employed here mostly assess memory and are largely reliant

on temporal lobe function while the 5CSRT assesses cognition broadly relying on more cortical regions and striatum (Robbins 2002).

In addition to the evaluation of cognitive function, systemic and brain exposures to DTX were also assessed. Our results demonstrate an influence of administration schedule on DTX brain exposure. In the intermittent regimen group, the DTX brain exposure profile is in agreement with that observed in plasma. Previous research shows that DTX is quickly absorbed from the peritoneal cavity after injection, with peak plasma concentrations occurring within 1 h before being rapidly eliminated from the systemic circulation over 8–12 h (Shimada et al. 2005; Yokogawa et al. 2004). Accordingly, peak DTX brain concentrations were observed shortly after intermittent treatment, followed by a rapid decrease; by 48 h post-injection, DTX was not detected in the brain. In contrast, in the sustained treatment group, the administered DTX-$PoLi_{gel}$ provided a controlled, continuous release of DTX, such that plasma concentrations were maintained for over 29 days. However, the sustained systemic DTX levels did not result in sustained DTX concentrations in the brain. Although DTX brain levels were measurable in the first 9 days, there was no DTX detected in the brain thereafter. It is thus likely that drug transfer into the brain was altered during the sustained treatment period.

DTX is a substrate of the multidrug resistance protein P-gp (Malingre et al. 2001; Wils et al. 1994) which is highly expressed in brain capillary endothelial cells. As an efflux pump, P-gp exhibits high efficiency in limiting DTX entry into the CNS (Gottesman and Pastan 1988; Hennessy and Spiers 2007). Moreover, P-gp is an inducible protein and various xenobiotics can initiate P-gp upregulation (Lee and Bendayan 2004). Our results indicate that both intermittent and sustained DTX treatment led to induction of P-gp. P-gp induction was seen in the sustained DTX treatment group throughout the 29-day study period, whereas P-gp expression increased rapidly but returned toward baseline within 9 days after intermittent doses. Therefore, the continuous induction of P-gp as well as low systemic concentrations likely limited DTX brain accumulation after a period of sustained administration. The low or undetectable levels of DTX in the brain may also explain why markers of autophagy and astrocyte activation were not found to be affected in the sustained group on day 29.

Apoptotic cell death has generally been accepted as the predominant cellular response to DTX chemotherapy (Mhaidat et al. 2007); therefore, it was logical to assess whether apoptosis was induced in the brains of our DTX-treated mice. Unexpectedly, immunodetectable levels of activated caspase-3 activity, a marker of apoptosis, were not significantly increased in the DTX-treated mice at 24 and 48 h after DTX treatment. While increased caspase-3 activity was not detected, it is important to note that assessing apoptosis at 24 and 48 h post-treatment could potentially miss the crucial time at which significant CNS cell death occurs. In contrast, significantly elevated autophagy activation, as measured by immunodetectable levels of activated LC3, was detected at 24 h after each injection in the intermittent treatment group. A strong trend towards increased levels of activated LC3 was also seen in the sustained treatment group, but this did not reach significance. Autophagy is both a protective cellular mechanism as well as a forerunner to cellular death depending on the situation and stage in the pathological process (Kroemer and Levine 2008; Tsujimoto and Shimizu 2005; Zhang et al. 2008). Although it is not clear whether the increase in autophagy activity observed here is a precursor to cell death or a protective process, systemic DTX treatment appears to induce CNS cellular stress consistent with levels of DTX brain exposure.

We subsequently measured astrocyte activation to further investigate potential DTX-induced injury to the CNS. Astrocytes are essential for neuronal function (Seifert et al. 2006) However, astrocytes also react to various insults to the CNS, leading to the activation of astroglia or astrogliosis (Eng and Ghirnikar 1994), which is characterized by the upregulation of GFAP, an intermediate filament protein (O'Callaghan 1991). In this study, GFAP levels were significantly increased in both treatment groups, and levels of activated astrocytes appeared to be consistent with DTX brain concentration exposure profiles according to treatment regimen. Specifically, transitory increases in activated astrocytes were detected in the brains of intermittently treated mice after each injection, while prolonged astrocyte activation (9 days after administration) was observed upon sustained DTX. Likewise, a pronounced increase in GFAP staining was seen in the hippocampal region of the DTX-treated mice. As the hippocampus is important for learning and memory, DTX-induced activation of astrocytes within the hippocampal sections could be partially responsible for the detrimental effects on MWM and NOR performance. While reactive astrocytes demonstrate a protective role in acute CNS injury, long-lasting astrocyte activation prevents axonal regrowth and has been implicated in a range of neurodegenerative disorders (Blackburn et al. 2009). Therefore, transient and prolonged activation of astrocytes following DTX administration suggests that both intermittent and sustained DTX administration could have profound effects.

Several limitations are worth mentioning. Firstly, the observed cognitive changes were modest, which suggests a lack of test sensitivity and a need for employing higher DTX doses. Secondly, quantitative brain toxicity analyses were conducted on whole brain samples, yet the cognitive tests employed were highly dependent on temporal lobe function. Although overall cellular stress within the brain, as indicated by increased autophagy and astrogliosis, is likely to negatively impact cognitive function, regional differences in sensitivity to neurotoxicity can occur, and it is therefore difficult to clearly link

these markers of CNS damage to performance. Nevertheless, immunohistochemical analysis of the hippocampus, which is located in the temporal lobe and plays an important role in spatial memory and cognition, confirmed astrocyte activation and cellular stress in this region in DTX-treated mice. Finally, although DTX brain concentrations and brain toxicity analyses delineate distinct consequences of intermittent and sustained DTX treatment, the effects induced by the two treatment strategies in the measures of cognition employed here were similar. This suggests that the sensitivity of the cognitive tasks employed or the timeline over which cognitive function was assessed needs to be further explored.

Overall, the present study provides strong and original evidence for DTX-induced toxic effects in the brain. These results show that DTX is able to penetrate through the BBB after i.p. administration and that DTX brain exposure profile may be influenced by dosing regimen. Furthermore, levels in the brain were associated with elevated markers of CNS stress, namely autophagy and astrocyte activation, indicating a plausible correlation between DTX dosing regimen and toxicity profiles in the brain. As such, rodent brains in the intermittent group were under substantial stress for a short period of time (within 48 h after each injection), while sustained DTX treatment resulted in a relatively prolonged stress but with lower intensity. In contrast, the cognitive effects of each of the treatment regimens were largely indistinguishable. Although we hypothesized that the lower systemic concentrations associated with sustained treatment would cause a lesser degree of impact on cognitive function, both intermittent and sustained DTX led to impaired object recognition and spatial reference memory shortly after treatment. As the same total cumulative dose of DTX was given to each treatment group, this suggests that total dose of DTX rather than peak brain concentrations according to DTX dosing schedule was more influential on subsequent cognitive function.

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