



Short communication

The chemotherapy agent oxaliplatin impairs the renewal of fear to an extinguished conditioned stimulus in rats[☆]Melissa J. Sharpe^b, Joanna E. Fardell^{a,d}, Janette Vardy^{c,d}, Ian N. Johnston^{a,*}^a School of Psychology, Brennan MacCallum Building (A18), University of Sydney, NSW 2006, Australia^b School of Psychology, University of NSW, Australia^c Sydney Cancer Centre, University of Sydney, Australia^d Cancer Institute New South Wales, Australia

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ABSTRACT

Recent evidence has shown that diverse chemotherapy agents can induce cognitive impairments and neurotoxic damage to the central nervous system. Oxaliplatin (OXP), a platinum compound, has been linked with acute and chronic peripheral neuropathies. This study explored the cognitive impacts of OXP in the rat with a fear conditioning procedure. 10 days prior to conditioning and testing, rats received an intraperitoneal injection of OXP (12 mg/kg). On the first day of conditioning, the rats were conditioned to two CSs (CS-ren and CS-ext) in one set of chambers (context A). They then received three tests on separate days. First, the rats were assessed for contextual fear conditioning in context A. Next, the CSs were presented 20 times in a new context (B) until fear conditioning had extinguished. Finally, one of the CSs (CS-ext) was tested again in the extinction context (B), and the other (CS-ren) presented in a new context (C). Results showed that OXP had no effect on the ability of rats to express fear to the conditioning context (A), or on the expression and extinction of conditioned fear to either CS when presented in a second context (B). However, the administration of OXP did impair the ability of rats to renew levels of conditioned fear to CS-ren when this CS was presented in a novel context (C) following extinction. This profile of impairment is consistent with hippocampal damage, and may also involve frontal cortical, amygdalar and thalamic regions important for context discrimination and the contextual modulation of behaviour.

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It is estimated that up to 75% of patients will experience cognitive impairment during and immediately after chemotherapy treatment, and a smaller subset (17–35%) will experience persistent deficits lasting years after treatment [1,2]. An emerging consensus from these studies is that chemotherapy is associated with mild, diffuse impairments in attention, verbal and visual memory, processing speed and executive function [3]. This is supported by evidence from neuroimaging studies that have correlated impaired performance on tasks involving memory and executive functioning with alterations in brain morphology and activation patterns in areas important for these tasks, such as the hippocampus and pre-frontal cortices [4,5].

Moreover, numerous studies have demonstrated that administering cytotoxic agents to laboratory rodents produces impaired performance in tasks requiring short- or long-term memory, or rule

learning. This approach has been used to test the negative cognitive and central neurotoxic effects of methotrexate (MTX) [6–14], 5-fluorouracil (5-FU) [7,15–19], MTX in combination with 5-FU [7,20], cyclophosphamide [18,21,22], doxorubicin [18,23–25], cyclophosphamide in combination with doxorubicin [26], paclitaxel [18], cisplatin [15,27], vincristine [27–29] and cytosine arabinoside [15,30]. Rodent models provide an ideal avenue for investigating the specific causal relationship between chemotherapy and cognitive function as they provide significant control over factors that prove problematic for human research in a clinical setting. The animals are free of cancer and other diseases, do not receive other medications (such as hormone therapy), or treatment for other comorbidities, and the role of other co-existing psychological factors that are prevalent in people, such as the stress of being diagnosed with cancer, can be largely ruled out. Moreover, the behavioural models for studying learning and memory in rodents are well established, and the neural systems important for performance in these models are well described. Thus, accompanied by the greater array of genetic and pharmacological tools for investigating neural function in rodents, this provides an ideal method for examining how chemotherapy impairs cognitive performance.

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* Corresponding author. Tel.: +61 2 9351 4353; fax: +61 2 9036 5223/9351 2603. E-mail address: i.johnston@sydney.edu.au (I.N. Johnston).

The aim of the present study is to examine whether oxaliplatin (OXP) causes memory and learning impairments in rats. OXP works by reacting with DNA to create DNA adducts, which block DNA synthesis and induce apoptotic cell death in rapidly dividing cells and cancer cells. It is currently being used in the treatment of solid tumours, such as colorectal cancer [31]. However, there are reasons to suspect that OXP may produce cognitive impairments. Other platinum-based compounds, such as cisplatin, produce central neurological damage [15,27]. Moreover, OXP has a well-described neurotoxic effect resulting in an acute hypersensitivity of peripheral nerves in most patients, and a chronic cumulative sensory neuropathy in a large subset of patients [32,33].

This study examined the effect of OXP in contextual fear conditioning in rats, a widely used model for studying the neural mechanisms of learning and memory [34]. Briefly, these experiments involve placing a rat into a conditioning chamber (the context; CX) and then presenting a brief audio and/or visual stimulus (the conditioned stimulus; CS) followed immediately with a mild but startling footshock (the unconditioned stimulus; US). The next time the rat experiences the CS, it will display its natural defensive behaviour and become immobile or freeze. CS fear conditioning (CSFC) is a relatively simple and rapid process requiring activity in the amygdala, and the medial prefrontal cortex (mPFC) [35]. In addition, the rat also learns to display fearful behaviour to the conditioning context independently of the CS. However, context fear conditioning (CXFC) requires the integration of diffuse spatial cues and is a slower process. While it requires activity in similar prefrontal and amygdalar neurocircuitry important for CSFC, CXFC is specifically sensitive to disruption of hippocampal function [36,37]. Moreover, given that prior exposure to the conditioning context prevents impairment of CXFC by hippocampal disruption [38], it is thought that the role of the hippocampus is not so much to associate the diverse contextual cues with the US but rather to learn about the relationships between the contextual cues themselves and to then consolidate them into long-term memory as a representation [39]. It is this representation that is then thought to become associated with the US. Thus, CXFC is often used as a method to study hippocampal-dependent spatial learning and long-term memory processes [34]. Given that the memory impairments induced by chemotherapy have been associated with altered hippocampal and prefrontal cortex morphology in people [4], this paradigm has been used previously to study the effect of chemotherapy on memory in rodents [8,13,16,26]. Indeed, previous work in this laboratory demonstrated that the sole administration of OXP or 5-fluorouracil did not result in deficits in a simple contextual fear conditioning paradigm. However, when these agents were administered in compound, a deficit was observed in CXFC but not CSFC [6,40].

We decided to extend these initial findings and examine whether OXP alone impairs learning about hierarchical relationships between the CX, the CS and the US. This is especially important in extinction, when the conditioned response to the CS is gradually reduced by repeated presentations of the CS without the US. Several lines of evidence suggest that extinction of a conditioned response to a CS involves learning to inhibit the conditioned responses normally elicited by the CS rather than simply erasing the original learning. For example, the original conditioned response may spontaneously recover when the CS is presented some time after extinction [41]. More recent evidence shows that the behavioural inhibition learned during extinction is context specific. That is, when an animal is presented with an extinguished CS in a different context, there is renewed expression of fear to the CS (the renewal effect) [42]. In the extinction context, it is thought that the hippocampus activates reciprocal circuits in the mPFC and/or amygdala that inhibit conditioned responding, but when the CS is experienced in a different context, the hippocampus stimulates different mPFC and amygdalar circuits that facilitate responding

[43,44]. Thus, by examining the renewal of a fear response to an extinguished CS in a new context after extinction, more subtle effects of OXP administration on learning and memory may be revealed than in a simple CXFC paradigm.

16 experimentally-naïve male Sprague-Dawley rats from ARC Perth, West Australia, weighing between 327 and 422 g were used. Rats were 92 days old when testing began and housed in groups of four in white plastic tubs measuring 26 cm × 59 cm × 37 cm (height × length × depth). Rats were kept in colony rooms using 12 h light/dark cycles beginning at 08:00 h. All testing was conducted during light cycles. Room temperature was maintained between 19 and 22 °C, and the animals received unrestricted access to food and water. Three different conditioning and testing contexts were used in this experiment (A, B and C). Context A was used for conditioning and consisted of four 31 cm × 30 cm × 25 cm (height × length × width) chambers with a front hinged door and back Perspex panel, with sidewalls and ceiling of aluminium. The flooring consisted of 16 stainless steel rods (6 mm diameter), spaced 12 mm apart. During conditioning, the scrambled shock USs were delivered to the metal floorings of context A via a constant current generator. The two conditioned stimuli consisted of a 1500 Hz tone or white noise presented at 74 dB against a 68 dB background via speakers mounted on the wall behind the chambers. These stimuli were counterbalanced so that half the animals in each group had the tone as the control extinction-only CS (CS-ext) and the white noise as the renewal CS (CS-ren), and vice versa for the other rats. Contexts B and C were used for testing extinction and renewal to the two CSs, and consisted of two different types of chambers that were counterbalanced within and between groups. One of those contexts consisted of two shuttle boxes. Doorways in the middle of each box were blocked, creating four chambers. These chambers consisted of Perspex hinged ceiling and walls, with aluminium walls separating the two chambers in each shuttle box. Each chamber had dimensions of 34 cm × 29 cm × 26 cm (height × length × width). The flooring consisted of 15 stainless steel rods. The third testing context consisted of four Perspex 600 mm × 150 mm (height × diameter) cylinders with open tops. A large cardboard shield separated each cylinder to prevent the rats from seeing each other. Behaviour was recorded by a camera mounted opposite the chambers.

Rats were injected with either OXP (Spirit Pharmaceuticals, Australia; 12 mg/kg i.p.; $n=8$) or equivolume saline (SAL; $n=8$) injection. The OXP dose was chosen as it reflects a dose equivalent to that achieved cumulatively in a clinical setting. Further, Ling et al. [51] found that rats given a 12 mg/kg of OXP exhibited peripheral neuropathies similar to those described in patients. Training and testing took occurred 10 days later and took place over 6 days. The design of the experiment is shown in Fig. 1. On the morning of the first day, the rats were placed in context A for 2 min before receiving the first of four CS presentations. The rats received two 10-s presentations of CS-ext and two 10-s presentations of CS-ren, with an intertrial interval (ITI) of 60 s. All CS presentations were followed by a 1-s 0.4 mA shock. CS-ext and CS-ren presentations alternated within the conditioning session, and the order of presentation was counterbalanced between the groups. The rats were removed from the conditioning chamber 30 s after the last CS. Context fear testing occurred 24 h following conditioning. The rats were placed back in context A for 10 min, and fear to the conditioning context (A), in the absence of the CS or US, was observed. The CS Extinction tests occurred on the afternoons of days 3 and 4. On each day, the rats were placed in context B and tested for fear to one CS. The order of testing of CS-ext or CS-ren was counterbalanced within and between groups. In each test, the rats received twenty 10-s CS presentations, separated by a 30-s ITI, with the first CS being presented 2 min after being placed in context B. On days 5 and 6, the rats were tested for renewal of the extinguished CSs. On one day,

Day	1	2	3 and 4	5 and 6
GROUP	CONDITIONING	CONTEXT TEST	CS EXTINCTION	CS RENEWAL
SALINE	A: CS-ext (+), CS-ren(+)	A: (-)	B: CS-ext (-) and B: CS-ren (-) (on separate days)	B: CS-ext (-) and C: CS-ren (-) (on separate days)
OXP				

Fig. 1. Experimental design used in this study. A, B, and C refer to three different types of conditioning or testing contexts counterbalanced between groups. CS-ext and CS-ren were either a 10-s tone or a white noise conditioned stimulus (CS) counterbalanced between groups. (+) indicates that a 0.4 mA, 1-s footshock unconditioned stimulus (US) followed the CS, (-) indicates that no shocks occurred. 10 days prior to conditioning, the rats were treated with oxaliplatin (OXP; 12 mg/kg i.p.) or equivalent saline (SAL). On the day of conditioning, all rats were placed into context A and received two presentations of CS-ext and two presentations of CS-ren in the same session, with each CS followed by the US. During the Context test, the rats were placed in context A with no presentations of either CS or US. During the Extinction and Renewal tests, CS-ext and CS-ren were tested on separate days. CS-ext indicates that the CS was tested in the same context (B) during the Extinction and the Renewal tests, CS-ren indicates the CS that was tested in one context (B) in the Extinction test, and a novel context (C) in the Renewal test.

the rats were placed back in the extinction context (B) and given 20 presentations of the control extinction CS, CS-ext, in the same manner as in extinction. On the other day, the rats were placed in the novel context (C) and given 20 presentations of the renewal CS, CS-ren, in the same manner as in the Extinction test. The order of Renewal testing was counterbalanced between and within the groups. Thus, CS-ext was tested in the same context as extinction, and CS-ren was tested in a novel context giving an ABB vs ABC within-subject renewal design. All procedures were reviewed and approved by the University of Sydney Animal Ethics Committee.

Freezing served as the index of conditioned fear and was defined as the absence of all movement except respiration. Using a time sampling procedure, a blind observer rated the rat as freezing or active every 10 s for the Context test, or every 2 s during CS presentations in the CS Extinction and CS Renewal tests. For the Context test, the percentage of observations that the rat was assessed as freezing from all observations during the test was divided into two 5-min sampling periods. The results from the Context fear test were analysed by two-way repeated measures ANOVA for a main effect of time and of OXP. For the CS tests, the level of freezing was calculated as the percentage of observations the rat was assessed as freezing during each CS. The results from the CS Extinction test were averaged into five-trial bins and analysed by repeated measures ANOVA for a main effect of OXP, a main effect of CS type (CS-ext vs CS-ren), and a main effect of CS presentation within the test session. The renewal of responding to a CS following contextual change is transient and should reflect an increase in responding relative to the end of the previous extinction phase. As such, renewal of freezing to an extinguished CS after a change in context was determined by comparing the change in freezing to CS-ext and CS-ren from the end of the CS Extinction test to the beginning of the CS Renewal test. Thus, the percentage change in freezing from the average of the last five trials of the Extinction test was calculated for the average of the first five CS presentations in the Renewal test for both CS-ext and CS-ren. These percentage change scores were then analysed by ANOVA for main effects of OXP and for CS type (CS-ext vs CS-ren). Significance was determined when $p < 0.05$. For post-hoc contrasts, significance was maintained at $\alpha = 0.05$ with Scheffé's procedure.

The results of the Context fear test in context A are shown in Fig. 2i. A two-way ANOVA revealed a non-significant trend towards a main effect of time, suggesting a reduction in freezing during the CX test, $F(1, 14) = 4.480$, $p = 0.053$. However, there were no significant between-group differences in overall levels of freezing during the test, $F(1, 14) = 0.224$, $p > 0.05$, or significant interaction between time and group, suggesting any reduction in freezing between the two points did not differ as a function of treatment $F(1, 14) = 0.068$, $p > 0.05$. Fig. 2ii shows the results of the CS Extinction test in context B. The mean percentage freezing for CS-ext and CS-ren significantly reduced across the 20 presentations in the CS Extinction test in

context B, $F(3, 42) = 27.212$, $p < 0.001$. However, there was no significant difference between the OXP and SAL treated rats in the level of conditioned fear to CS-ext or CS-ren, or in the rate of extinction ($F_s < 0.8$). Fig. 2iii shows the results of the first five trials of the Renewal test, where CS-ext was presented again in context B while CS-ren was presented in the novel context C. There was a large increase in freezing to CS-ren, but not CS-ext, in the SAL treated rats, but there was very little increase in freezing to CS-ext or CS-ren in the OXP treated rats. While there was no main effect of CS type, there was a non-significant trend toward a main effect of OXP, $F(1, 14) = 3.901$, $p = 0.068$, and there was a significant interaction between CS type and group, $F(1, 14) = 5.149$, $p < 0.05$. Post-hoc analyses of the Renewal test failed to reveal differences between OXP and SAL treated rats for CS-ext, $F(1, 14) = 0.117$, critical $F = 4.6$, but revealed a significant difference for CS-ren, $F(1, 14) = 8.575$, critical $F = 4.6$.

The first major result of this study is that OXP did not have an anterograde effect on context fear conditioning, consistent with our previous findings [6,40]. Anterograde impairments in CXFC have been observed in rats treated with chronic cyclophosphamide and doxorubicin [26] or with chronic 5-FU [16]. Similarly, Seigers et al. [13] demonstrated a retrograde amnesic effect of acute high dose MTX on the conditioning of fear to a CX when administered immediately after conditioning. These groups argued that this indicates that chemotherapy impairs hippocampal-dependent spatial learning processes important for CXFC, and is supported by reductions in hippocampal cell proliferation and blood vessel density following treatment [11–13,16]. Furthermore, the administration of other chemotherapeutic agents has induced impairments in other spatial memory tasks (i.e., the water maze or object location recognition) [11–13,16]. Hippocampal impairment has also been observed in other laboratories after MTX [9,10], 5-FU [18,19], cyclophosphamide [18,21], doxorubicin [18], or paclitaxel [15,18]. On the other hand, Gandal et al. [8] observed that mice given chronic administration of a combination of MTX and 5-FU showed increased fear to a context paired with an aversive US. However, Gandal et al. [8] also observed higher levels of immediate post-shock freezing, which does not require an intact hippocampus, and they also pre-exposed the mice to the conditioning room, a procedure that may reduce the importance of hippocampal-dependent processing [39].

The results of the present study suggest that an acute administration of OXP did not impair performance in CXFC. Thus, if OXP had an impact on hippocampal function, it was not of a sufficient extent to disrupt the acquisition of CXFC. Interestingly, previous work has shown that complete bilateral neurotoxic lesions of the dorsal hippocampus prior to conditioning do not affect CXFC in rats [45]. It has been argued that animals without a functioning hippocampus compensate for their failure to normally integrate contextual cues

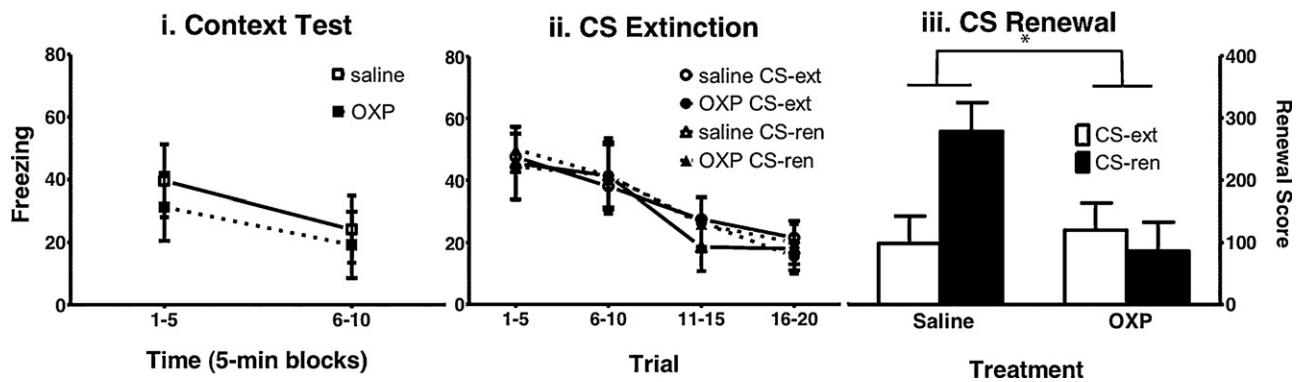


Fig. 2. The effect of oxaliplatin (OXP) on fear conditioning to two stimuli (CS-ext and CS-ren) conditioned in context A. (i) Percentage of time spent freezing when the rats were returned to context A 24 h after conditioning. (ii) Extinction of freezing to CS-ext and CS-ren in context B after 20 presentations of each stimulus. (iii) Change in freezing from the last five trials of the extinction test for CS-ext, which was presented in the same context as extinction (context B), and CS-ren, which was presented in a novel context (context C). * indicates a significance interaction between the type of CS tested and drug treatment, $p < 0.05$.

by learning to directly associate individual discrete elements of the context with a US via simple associative mechanisms in other brain regions [37,45]. The possibility remains that OXP did damage hippocampal processes important for normal context learning, but that recruitment of simple associative mechanisms may have compensated for this impairment.

Indeed, the administration of OXP also failed to impair conditioning or extinction to a CS, indicating that processes mediating learning between simple discrete cues were still intact. The initial level of fear expressed to the CS, the rate of extinction of the CS in the first Extinction test, and the levels of fear of CS-ext in the second Extinction test were comparable between OXP- and SAL-treated rats. Thus, it would seem that the circuits in the amygdala and mPFC important for the learning and expression of CSFC, for mediating the extinction of CSFC within an extinction session, and for preserving extinction between extinction sessions were not extensively impaired by OXP.

However, in addition to examining CSFC and CXFC, we also assessed whether OXP would affect whether the rats could learn to use the background contextual cues to modulate the expression of CSFC after extinction. We observed that SAL-treated rats expressed high levels of conditioned fear to an extinguished CS when the background contextual cues at test differed from that of extinction. OXP-treated rats, on the other hand, failed to exhibit renewed levels of responding to the CS when presented in a novel context after extinction. This suggests that OXP impaired the processes that allow an animal to modulate responding to an extinguished CS depending on which context it was experienced in. One possibility is that OXP impaired the ability of the hippocampus to form an integrated representation of the extinction context, rendering the rats unable to learn where extinction occurred. However, as was noted above, OXP treated rats were able to learn to associate the conditioning context with the US suggesting that the processes important for the initial learning about a context were not significantly impaired.

Another possibility is that OXP-treated rats may have suffered a different form of impairment in spatial processing and memory rendering them unable to discriminate the conditioning and extinction contexts and to detect that the CS was presented in a new context. In contrast to the initial development of contextual representations, context discrimination is thought to require more complex match-mismatch analyses of the features of current and expected contexts based on past experience [46]. Analyses of this nature are thought to be dependent on complex processing in the hippocampus, but also likely require extra-hippocampal structures including the mPFC, amygdala and mediodorsal thalamus [47,48].

Indeed, other studies in our laboratory [6,40] show that object recognition memory is impaired in OXP-treated rats.

A third possibility is that OXP-treated rats may have been able to detect the contextual change, but may have acquired impairment in the processes important for flexibly modifying behaviour when the context changes. Renewal of conditioned fear in response to context change is due to hippocampal modulation of reciprocal circuits in the mPFC and amygdala important for regulating responding to a CS [44]. Given that CSFC and extinction occurred normally in OXP-treated animals, it is unlikely that OXP extensively damaged these circuits. Rather, the possibility remains that OXP may have caused diffuse damage across the hippocampus, the mPFC and/or the amygdala, impairing communication between them and preventing the effective modulation of responding to a CS in response to a change in context.

Exactly how OXP might cause these impairments is unknown. OXP has well-described peripheral neurotoxic effects [32,33], and penetrates the blood-brain barrier and accumulates in the CNS [49]. The peripheral neuropathic effects of OXP appear to be linked to acute effects of neuronal voltage-gated sodium channels and calcium signalling, and chronic effects on dorsal root ganglion cellular metabolism and axonal transport [32]. Moreover, OXP-induced neuropathies are also associated with changes in PKC-activated p38MAPK activity in supraspinal regions of the CNS important for pain sensation [50]. However, the effect of OXP in regions important for cognition has not been explored. Despite these ambiguities, the present findings suggest that the administration of chemotherapeutic compounds have a mild and subtle effect on the learning and memory processes necessary to facilitate the contextual control of behaviour. The profile of impairment is consistent with hippocampal damage, and may also involve mPFC, amygdala and thalamic regions important for context discrimination and the modulation of behaviour.

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