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Paraquat and psychological stressor interactions as pertains to Parkinsonian co-morbidity

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ABSTRACT

A number of epidemiological and experimental studies have implicated the non-selective herbicide, paraquat, in the development of sporadic Parkinson's disease (PD). While preclinical research has focused mainly on elucidating the nigrostriatal effects of paraquat, relatively little data are available concerning non-motor brain systems and inflammatory immune processes (which have been implicated in PD). Hence, in the present study, we sought to take a multi-system approach to characterize the influence of paraquat upon extra-nigrostriatal brain regions, as well ascertain whether the impact of the pesticide might be enhanced in the context of chronic intermittent stressor exposure. Our findings support the contention that paraquat itself acted as a systemic stressor, with the pesticide increasing plasma corticosterone, as well as altering neurochemical activity in the locus coeruleus, paraventricular nucleus of the hypothalamus, nucleus accumbens, dorsal striatum, and central amygdala. However, with the important exception striatal dopamine turnover, the stressor treatment did not further augment these effects. Additionally, paraquat altered inter-cytokine correlations and, to a lesser extent, circulating cytokine levels, and concomitant stress exposure modulated some of these effects. Finally, paraquat provoked significant (albeit modest) reductions of sucrose preference and weight gain, hinting at possible anhendonic-like or sickness responses. These data suggest that, in addition to being a well known oxidative stress generator, paraquat can act as a systemic stressor affecting hormonal and neurochemical activity, but largely not interacting with a concomitant stressor regimen.

1. Introduction

Epidemiological data have for some time now supported a link between Parkinson's disease (PD) and cumulative lifetime pesticide exposure (Tanner et al., 2011; Wang et al., 2011; Kamel et al., 2013), and it has been suggested that the pro-oxidant pesticide, paraquat, could be especially relevant for some of the neuropsychiatric symptoms of the disease (Kim et al., 2013). Besides its effect on the nigrostriatal system, exposure to paraquat in rodents has produced other neurotoxic biological characteristics associated with PD including microglia activation, Lewy-body like aggregates containing α-synuclein, pro-inflammatory cytokine expression, and oxidative stress via mitochondria complex inhibition or microglia activation (Litteljohn et al., 2011a; Baltazar et al., 2014).

Upon entry into the brain, paraquat is distributed across areas including the prefrontal cortex, hippocampus, olfactory bulbs, and the substantia nigra pars compacta (SNc) (Peng et al., 2007). The pesticide was also found to induce several PD-like non-motor behavioural deficits, including olfactory dysfunction (Czerniczyniec et al., 2011), anxiety-like symptoms (Litteljohn et al., 2009; Czerniczyniec et al., 2011; Campos et al., 2013), and memory impairment (Chen et al., 2010). Nonetheless, it has yet to be determined whether paraquat can induce anhedonic-like behaviour in rodents, and the need for understanding the pesticide's

Abbreviations: 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, serotonin; ANOVA, analysis of variance; CIS, chronic intermittent immobilization/social defeat stressor; DA, dopamine; DOPAC, 3,4-Dihydroxyphenylacetic acid; EDTA, ethylenediaminetetraacetic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; HPLC, high-performance liquid chromatography; HVA, homovanillic acid; IFN-γ, interferon-γ; IL, interleukin; KO, knockout; LC, locus coeruleus; LLOQ, lower limit of quantification; MCP, monocyte chemoatrractant protein; MHPG, 3-methoxy-4-hydroexyphenylglycol; MIP, macrophage inflammatory protein; NE, norepinephrine; PD, Parkinson's disease; PVN, paraventricular nucleus; TNF-α, tumour necrosis factor-alpha.

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effects on extra-nigrostriatal brain regions should be broadened.

In addition to biological insults, it is conceivable that psychologically relevant stressors could affect the primary motor symptoms and neurodegenerative process (Urakami et al., 1988; Metz, 2007; Kibel and Drenjancević-Perić, 2008; Smith et al., 2008), as well as non-motor or co-morbid neuropsychiatric manifestations in PD patients. For instance, major life events influenced the development of depression among PD patients (Rod et al., 2013), and psychological therapies have proven to be effective in reducing depression and anxiety symptoms in PD patients (Yang et al., 2012; Schrag et al., 2001; Hurt et al., 2012; Whitworth et al., 2013).

In the present investigation we sought to assess the individual and combined effects of the PD-linked pesticide, paraquat, and a psychologically relevant chronic intermittent immobilization/social defeat stressor (CIS) challenge. We were particularly interested in further characterizing the influence of paraquat upon extra-nigrostriatal brain regions and ascertaining whether the disparate classes of stressors (chemical vs. psychological) would interact to influence hedonic behaviour. It was also of interest to determine whether any such effects would be accompanied by changes in stressor hormone levels (corticosterone), central neurotransmitter activity and immune messengers (circulating cytokines). Specifically, it was hypothesized that chronic systemic paraquat administration and concurrent CIS exposure would provoke physiological and behavioural changes consistent with a depressive-like phenotype, as expected in a sizable portion of PD patients.

2. Materials and methods

2.1. Animals and general experimental design

Male C57BL6/J mice were obtained at 6–7 weeks of age from The Jackson Laboratory (Bar Harbor, ME, USA) and acclimated to our vivarium for 2 weeks. Animals were singly housed in standard polypropylene cages (27 × 21 × 14 cm) and maintained on a 12-h light/dark cycle with lights on at 08:00 h. A diet of Ralston Purina (St. Louis, MO) mouse chow and water was provided ad libitum, and room temperature was maintained at ~21 °C. One week prior to the commencement of the study, mice were randomly assigned to one of four experimental conditions (No stress/Saline; No stress/Paraquat; Stress/Saline; Stress/Paraquat) and sucrose preference training initiated (n = 10–12). A further 8 mice comprised the testing-naïve negative control: except for behavioural testing (see below), these animals received identical treatment to the No stress/Saline mice. All animals were rapidly decapitated 2 h following the final paraquat or saline injection. In order to minimize the effects of diurnal variations, tests and procedures were carried out between the hours of 08:00 and 13:00. All experimental procedures were approved by the Carleton University Committee for Animal Care and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

2.2. Experimental treatments: paraquat and chronic intermittent stress

All mice received intraperitoneal injection with 10 mg/kg paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; Sigma Aldrich, St Louis, MO, USA) or physiological saline (Sigma Aldrich). Injections were administered twice a week for 6½ weeks (13 injections in total) on a regular interval basis. This paraquat dose is routinely used in our lab and has been shown to reliably induce nigrostriatal damage (~25–35% neuron loss) (Mangano and Hayley, 2009; Mangano et al., 2011). During the 30 min immediately preceding each injection, half of the animals were either socially defeated or physically restrained in semicircular Plexiglas tubes (4 × 12 cm) with tails taped to prevent turning. The social defeat paradigm involved introducing experimental mice into the home-cage of a significantly larger and more aggressive mouse (retired CD1 breeders from Charles River, QC, CAN). A mesh wire divider was inserted into the cage upon the first display of submissive behaviour (upright posture with belly exposed) or if excessive fighting occurred (continuous biting); this had the effect of physically separating the mice while still allowing for interactions between them (Audet et al., 2011). Stressor application followed a fixed alternating schedule such that each mouse in the stressor groups received one session per week of restraint and one of social defeat. On the day of sacrifice all animals in the stressor conditions received 30 min restraint immediately prior to the final paraquat or saline injection. Due to the nature of the stressor paradigm, the stressed animals were housed in holding rooms separate from, but otherwise identical to, their non-stressed counterparts.

2.3. Brain dissection technique

Following rapid decapitation, brains were excised and sectioned into sequential coronal slices using razor blades and a chilled stainless steel microdissecting matrix with adjacent slots spaced ~0.5 mm apart. Hollow biopsy needles were used to collect the dorsal striatum, paraventricular nucleus of the hypothalamus (PVN), locus coruleus (LC) and nucleus accumbens. All tissue samples were taken with reference to the mouse brain atlas of Franklin and Paxinos (1997). Samples were maintained in a homogenizing solution containing 14.17 g monochloroacetic acid, 0.0186 g disodium ethylenediamine tetraacetate (EDTA), 5.0 ml methanol, and 500 ml H_2O; and stored at −80 °C until determination of central monoamine and metabolite levels using high performance liquid chromatography (HPLC).

2.4. Plasma corticosterone assay

At the time of decapitation, trunk blood from all of the animals, including those in the behavioural testing-naive control group, was collected in tubes containing 10 μg EDTA. Samples were centrifuged (3000g for 8 min) and the plasma removed and stored in aliquots at −80 °C for later corticosterone determination with commercially available radioimmunoassay kits (ICN Biomedicals, CA, USA). Samples were assayed in duplicate within a single run to control for inter-assay variability; the intra-assay variability was less than 10%. Separate plasma aliquots were used for the cytokine determinations.

2.5. Plasma cytokine quantification

Circulating levels of 11 different cytokines were determined by multiplex analysis using the Luminex 100 suspension-based bead array system (Luminex Corp., Austin, TX) and a custom multiple cytokine detection kit (MILLIPLEX MAP Mouse Cytokine/Chemokine Kit, Millipore, Cat. #MPXMCYTO-70K). Each of the 11 cytokines assayed are listed in Table 1. The assay was performed according to the kit manufacturer's instructions (see www.millipore.com/userguides) and, unless otherwise indicated, all reagents were provided in the multiplex kit. Eight samples from each of the four treatment groups were run in duplicate; the remaining samples were singly run. Where applicable, results of duplicate sample determinations were averaged prior to the data being analysed. In cases where cytokine levels were so low as to be undetectable, samples were assigned a value of one-half the lower limit of quantitation.

Table 1
List of assayed plasma cytokines, brain monoamines and amine metabolites.

Analyte name/symbol	Full name
Cytokine	
IFN-γ	Interferon-gamma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL-1β	Interleukin-1-beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12 (p40)	Interleukin-12 (p40)
IL-12 (p70)	Interleukin-12 (p70)
IL-17	Interleukin-17
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1-alpha
TNF-α	Tumour necrosis factor-alpha
Monoamine/metabolite	
DA	Dopamine
DOPAC	3,4-Dihydroxyphenylacetic acid
HVA	Homovanillic acid
NE	Norepinephrine
MHPG	3 Methoxy-4-hydroxyphenylglycol
5-HT	Serotonin
5-HIAA	5-Hydroxyindole acetic acid

2.6. HPLC determination of central amine and metabolite concentrations

Regional brain levels of monoamines and their primary metabolites (see Table 1) were determined by HPLC in keeping with previously reported methods (Litteljohn et al., 2014). Tissue punches were homogenized by ultrasonic disruption (Sonic Dismembrator Model 100, Fisher Scientific) in the homogenizing solution in which they were initially frozen (with DHBA as an internal standard). The level of protein was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific 23225). Homogenized samples were centrifuged (12,000 rpm for 3 min at 4 °C), after which 50 μl of supernatant was injected, at a flow rate of 1 ml/min, into the automated HPLC system (Agilent 1100) with electrochemical detector (DECADE II SDC, Antec) and ZORBAX Eclipse XDB-C8 columns (Agilent: 4.6 mm inner diameter, 150 mm length, 5 μm particle size; thermostated at 40 °C); the oxidation potential was maintained at 0.60 V. The mobile phase comprised: 90 mM sodium phosphate monobasic, 1.7 mM 1-octanesulfonic acid, 50 mM EDTA, 10% acetonitrile, 50 mM citric acid (monohydrate), 5 mM KCL, and HPLC-grade water. Monoamine and metabolite concentrations were expressed relative to the protein content of the samples, and final results presented as ng/mg protein (Litteljohn et al., 2014).

2.7. Repeated sucrose preference testing

Baseline sucrose preference training was undertaken during the seven days leading up to the start of the experiment. Briefly, singly housed mice were presented with two identical 15 ml ball-bearing sipper tubes (introduced into the home-cage through spaces in the cage lid), one of which contained regular tap water and the other a 1% palatable sucrose solution. Animals were permitted ad-lib access to both of the bottles, which were weighed at the same time each morning and the amount consumed from each recorded. Bottles were rotated daily and across weeks to offset potential positional preferences. Sucrose preference was calculated according to the formula: Sucrose preference = [sucrose intake/(sucrose intake + water intake)] × 100. In this way, 24-h baseline sucrose preference levels were established for the animals prior to initiating any of the experimental treatments. Only those mice demonstrating a steady baseline sucrose preference (average sucrose preference over the final 2 baseline training days >75%) were used in the study; all but 1 mouse met or surpassed the predetermined cut-off value. Each week commencing at 60 min after the second of the two weekly injections/stressors, animals were permitted free access for 3 consecutive days to the water and sucrose solutions. To avoid capturing any acute toxic effects of the pesticide, recovery day data (corresponding to the 24-h period commencing the morning after the most recent treatment application) were collected and analysed.

Although we considered the repeated sucrose-testing paradigm to be non-invasive and minimally stressful, an additional cohort of animals was included to explicitly assess the degree of stress associated with the testing regimen. To this end, blood samples from the testing-naïve control mice were assayed for corticosterone concentration, and the data included in the between-groups analysis. It was decided *a priori* that a statistically significant difference in plasma corticosterone levels between the two non-stressed, saline-treated control groups (assessed via the two-sample t-test, $p < 0.05$) would be considered evidence of a testing-related stressor-like effect and, as such, would warrant inclusion of testing-naïve samples in (or at the minimum alter the interpretation of the results from) the subsequent cytokine and monoamine assessments. In fact, this was not the case following this analysis.

2.8. Statistical analyses

Data were analysed by 2 (*Injection*; saline v. paraquat) × 2 (*Stress*; no stress v. stress) ANOVAs followed by Fisher's planned comparisons ($p < 0.05$) where appropriate. Where applicable, repeated measures ANOVAs were conducted with *Time* as the 3rd independent variable. In instances where it was of interest to assess the linear relationship between outcome variables (e.g., cytokine network analysis), this was done via Pearson product moment correlations (r) and, in certain cases, coefficients of determination (r^2). As numerous correlations were conducted, the α level of statistical significance was set to <0.025, per the method of Poulter et al. (2010). In addition, we ran χ^2 analyses to determine whether the frequency of significant cytokine correlations differed between the treatment groups ($p < 0.05$). During the course of tissue dissection and HPLC analyses a few samples were lost. For the sucrose preference test, data points exceeding 2 standard deviations were deemed outlier values and omitted from the analyses. Data were evaluated using a StatView (version 6.0) statistical software package and visualized with GraphPad Prism 6 (La Jolla, CA). The latter program was also used for the χ^2 analyses.

3. Results

3.1. Plasma cytokine levels and inter-correlations were altered by paraquat and stress

As shown in Fig. 1A, paraquat increased the circulating concentrations of the haematopoietic colony-stimulating factor, GM-CSF ($F_{1, 35} = 4.75$, $p < 0.05$). None of the other assayed cytokine species were significantly affected by the pesticide treatment (with respect to absolute protein levels; data not shown). As it relates to the CIS treatment, IL-6 levels were significantly elevated overall following stressor exposure ($F_{1, 34} = 5.62$, $p < 0.05$), though the magnitude of this effect was quite small and the absolute levels of this cytokine rather low across all groups (Fig. 1A). In contrast, plasma levels of the IL-12 subunit, IL-12 (p40), were reduced in mice receiving the stressor treatment, irrespective of pesticide exposure ($F_{1, 32} = 4.24$, $p < 0.05$).

Fig. 1B shows the cross-correlations between the different cytokines among non-stressed (top panel) and stressed mice (bottom panel) as a function of saline (left matrices) and paraquat treatment

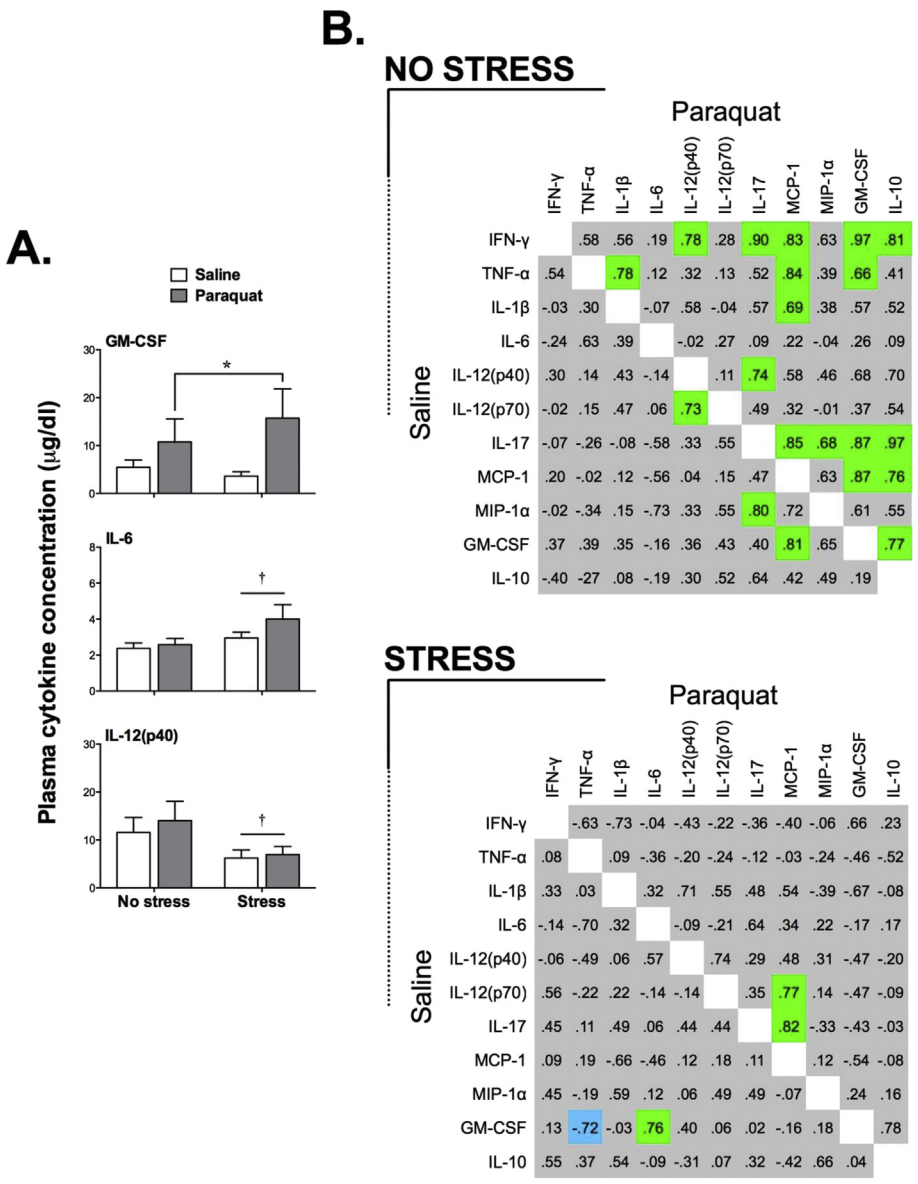

Fig. 1. Concentrations of select cytokines following treatment with paraquat and/or a chronic intermittent stressor (Panel A). Whereas the pesticide increased the circulating levels of granulocyte macrophage colony stimulating factor (GM-CSF), the stressor increased interleukin-6 (IL-6) and decreased IL-12 (p40) concentrations; no interaction effects were observed. Panel B shows the cross-correlations between the different cytokines among non-stressed (top panel) and stressed mice (bottom panel) as a function of saline (left matrices) and paraquat treatment (right matrices). Precise correlation values are presented within the individual squares of each matrix and the squares are colour-coded so as to better reflect the patterns of correlations: Green and blue indicate significant positive and negative correlations, respectively ($p < 0.025$), while grey denotes a lack of statistical significance (see text for additional details). *$p < 0.05$ relative to saline-treated mice (collapsed across the stressor treatment); †$p < 0.05$ relative to non-stressed mice (collapsed across the paraquat treatment).

(right matrices). Precise correlation values are presented within the individual squares of each matrix and the squares are colour-coded so as to better reflect the patterns of correlations: green and blue indicate significant positive and negative correlations, respectively ($p < 0.025$), while grey denotes a lack of statistical significance. Among the saline-treated animals, very few correlations were found to be statistically significant and their frequency (3 positive correlations in the non-stressed group, 1 positive and 1 negative correlation in the stressed condition) was unaffected by the stressor treatment ($\chi^2 = 0.21$, $df = 1$, $p = 0.65$). Yet, it should be noted that the stressor did abolish the few significant correlations that were observed among the non-stressed saline controls, and seemed to selectively influence correlations involving GM-CSF (Fig. 1B).

Following paraquat treatment, the frequency of significant cytokine correlations among the non-stressed mice increased from 3 (of a possible 66 correlations) to 17 ($\chi^2 = 9.96$, $df = 1$, $p < 0.01$), and all of these associations were positive and ranged in strength from weak ($0.4 < r^2 < 0.5$) to very strong ($r^2 > 0.9$). As can be seen in Fig. 1B, almost half (8 out of 17) of the significant correlations in the No stress/Paraquat condition involved IL-10 and GM-CSF, and these cytokines themselves demonstrated a moderate linear relationship ($r^2 = 0.59$). Perhaps even more striking was the marked difference in the profile of significant paraquat-associated cytokine correlations among the non-stressed vs. stressed mice. Whereas in the former group, paraquat clearly enhanced the rigidity of the peripheral cytokine network, no such effect was apparent in the latter group (relative to the stressor alone-treated mice) (Fig. 1B). In fact,

the stressor reduced the frequency of significant paraquat-associated cytokine correlations from 17 to 2 ($\chi^2 = 12.05$, $df = 1$, $p < 0.001$).

3.2. Paraquat increased plasma corticosterone concentrations as well as monoamine activity in the LC and PVN

There was no difference in plasma corticosterone content between the non-stressed, saline-treated control mice that underwent behavioural testing and the testing-naïve negative controls ($t_{14} = 0.68$, $p > 0.50$: 6.57 ± 0.66 *v* 5.76 ± 1.06). Accordingly, pooled data from these two groups were used for the between-groups corticosterone analysis ($n = 16$). The 2-way ANOVA indicated that plasma corticosterone concentrations were modestly but significantly increased following treatment with either paraquat or the stressor ($Fs_{1, 43} = 4.26$ and 4.52, respectively, $p < 0.05$). As depicted in Fig. 2A, while the interaction effect of Paraquat with Stress was not statistically significant ($p > 0.10$), levels of the stress hormone appeared to be highest among mice receiving the combination treatment.

In addition to augmenting plasma corticosterone, paraquat increased NE concentrations within the locus coeruleus (LC) ($F_{1, 33} = 6.62$, $p < 0.05$) and PVN ($F_{1, 27} = 33.31$, $p < 0.0001$) among non-stressed and stressed mice alike (Fig. 2B). In contrast, within these brain regions, paraquat did not significantly alter the levels of the primary NE metabolite, MHPG. While the PVN concentrations of 5-HT, 5-HIAA and DA were likewise unchanged following the pesticide treatment, accumulation of the DA metabolites, DOPAC and HVA, was significantly enhanced overall among the paraquat-treated animals ($Fs_{1, 27} = 7.37$ and 13.3, $p < 0.05$: 7.63 ± 1.17 *v* 13.56 ± 2.42 and 10.74 ± 1.15 *v* 15.38 ± 1.27, respectively).

The chronic intermittent stressor had comparatively less influence than paraquat on LC and PVN monoaminergic activity. Still, within the PVN, levels of both 5-HT ($F_{1, 27} = 9.34$, $p < 0.01$; see Fig. 2A) and HVA ($F_{1, 27} = 10.18$, $p < 0.01$: 11.14 ± 1.20 *v* 15.29 ± 1.22) were significantly elevated overall among animals undergoing the stressor treatment.

3.3. Altered dopaminergic activity within the nucleus accumbens and dorsal striatum following paraquat and stressor exposure

Within the reward-relevant nucleus accumbens, DA levels varied as a function of the significant interaction between Pesticide and Stress ($F_{1, 38} = 6.53$, $p < 0.05$). As shown in Fig. 3 and confirmed by the follow-up comparisons, while accumbal DA concentrations were diminished (to a similar level) by treatment with either paraquat or CIS ($p < 0.05$), combining the two insults did not lead to a further decrement in parent amine levels. Separate ANOVAs also revealed significant Pesticide × Stress interaction effects for DOPAC and HVA levels ($Fs_{1, 38} = 10.17$ and 8.77, respectively, $p < 0.01$). Whereas in the former case, pesticide exposure normalized the stressor-induced elevation of DOPAC, in the latter case paraquat interacted with the stressor treatment to enhance accumbal HVA accumulation ($p < 0.01$ compared to all other groups) (Fig. 3). The overall effect(s) of the experimental treatments on DA utilization in the nucleus accumbens can perhaps best be appreciated by examining the rate of DA turnover (i.e., the ratio of DA metabolites to DA); not surprisingly, this was also noted to vary as a function of a significant Paraquat × Stress interaction ($F_{1, 38} = 7.72$, $p < 0.01$). The post-hoc analyses confirmed that the pesticide and stressor each provoked a significant increase in the DA metabolite-to-parent amine ratio ($p < 0.01$ relative to the non-stressed, saline-treated controls).

Within the dorsal striatum, there were no significant effects of paraquat or the CIS treatment on DA concentrations (Fig. 4). However, higher HVA levels were observed among mice receiving the pesticide treatment ($F_{1, 37} = 7.04$, $p < 0.05$), and a significant Pesticide × Stress interaction was revealed for striatal DOPAC ($F_{1, 37} = 7.30$, $p < 0.05$). Specifically, mice that received either the paraquat alone or stressor alone treatments displayed diminished DOPAC concentrations relative to the non-stressed, saline-treated animals ($p < 0.05$). Contrastingly, DOPAC levels in the paraquat-plus-stressor-treated mice were unchanged compared to controls (Fig. 4). The pattern of paraquat-and-stressor-associated dopaminergic changes suggested that the combination treatment had the

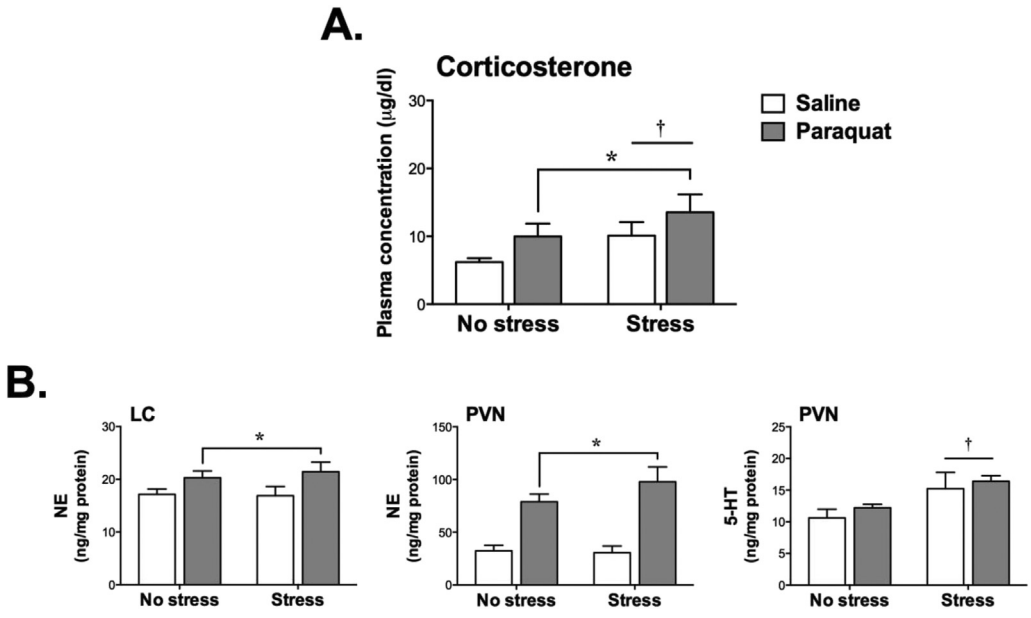

Fig. 2. Influence of paraquat and chronic intermittent stress on elements of the murine stress response. Both paraquat and stress significantly (but independently) increased plasma corticosterone levels (A). As shown in panel B, paraquat augmented norepinephrine (NE) concentrations within the locus coeruleus (LC) and paraventricular nucleus (PVN); in the latter region, stress also increased serotonin (5-HT) levels. *$p < 0.05$ relative to saline-treated animals; †$p < 0.05$ relative to non-stressed mice.

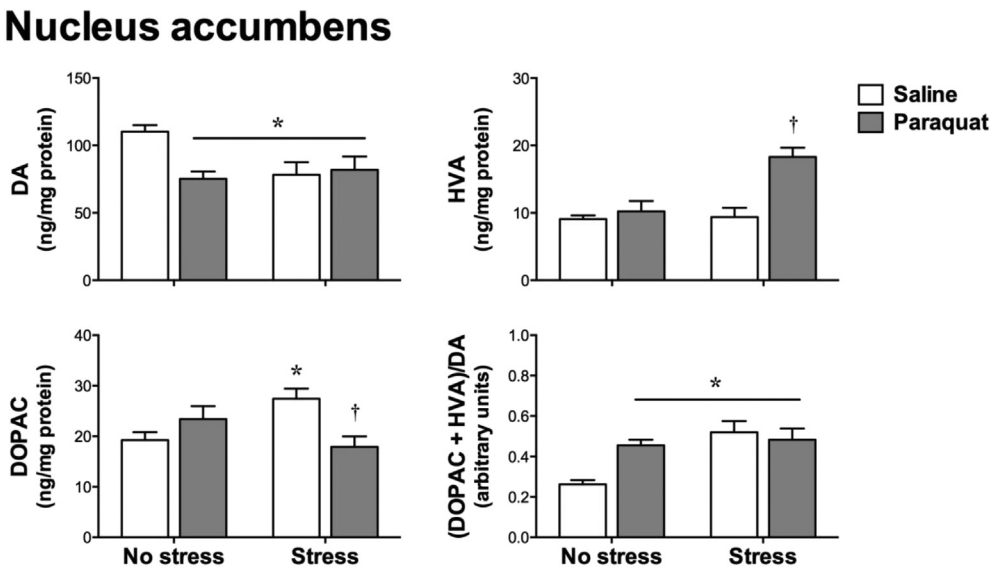

Fig. 3. Dopaminergic activity in the nucleus accumbens as a function of paraquat and stressor exposure. The non-stressed, saline-treated controls had higher dopamine (DA) levels (top left) and diminished DA turnover rates (bottom right) compared to all other treatment groups. Also depicted are the data for 3,4-Dihydroxyphenylacetic acid (DOPAC; bottom left) and homovanillic acid (HVA; top right) (see text for more details). *$p < 0.05$ relative to saline-treated, non-stressed controls; †$p < 0.05$ relative to saline-plus-stressor-treated mice.

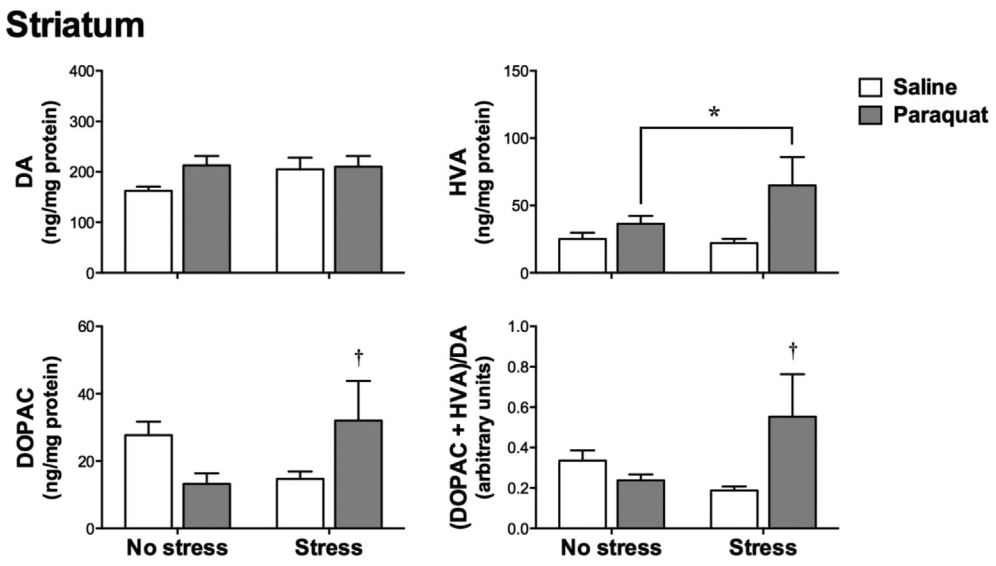

Fig. 4. Dopaminergic activity in the dorsal striatum as a function of paraquat and stressor exposure. Co-treatment with paraquat and stress had the effect of increasing dopamine (DA) turnover within the dorsal striatum (in the absence of altered parent amine levels). Also depicted are the data for striatal 3,4-Dihydroxyphenylacetic acid (DOPAC; bottom left) and homovanillic acid (HVA; top right) levels (see text for more details). *$p < 0.05$ relative to saline-treated mice (collapsed across stressor exposure); †$p < 0.05$ relative to saline-plus-stressor-treated mice.

effect of increasing DA turnover within the dorsal striatum, similar to what was previously seen in immobilization-plus-MPTP-treated mice (Urakami et al., 1988). This was borne out by the ANOVA, which revealed a significant Pesticide × Stress interaction effect on DA turnover ($F_{1,\ 37} = 5.70$, $p < 0.05$). As shown in Fig. 4 and confirmed by the post-hoc tests, only those animals co-treated with paraquat and stress displayed enhanced DA turnover in the striatum ($p < 0.05$).

3.4. Stressor and paraquat effects on sucrose consumption and weight gain

Although there was no significant main effect for the stressor treatment nor were there any significant interactions between any of the variables, the repeated measures ANOVA indicated that paraquat itself had the overall effect of reducing animals' preference for a palatable sucrose-containing solution ($F_{1,\ 33} = 5.55$, $p < 0.05$). Despite the lack of significant interaction effects of *Paraquat* with *Stress* or *Time*, from Fig. 5 (top) it is clear that the paraquat effect developed gradually over time and that the pesticide had its most pronounced effect in mice that were also exposed to the stressor. Indeed, the multiple comparisons indicated that the paraquat-plus-stressor-treated animals were the only ones to exhibit significant decrements in sucrose preference and this occurred at Weeks 4, 5 and 6 ($p < 0.05$ relative to saline-treated controls). Assessment of difference between baseline and Weeks 6, indicated that the stress alone group was identical to controls and the paraquat alone treatment caused only a 1.5% sucrose

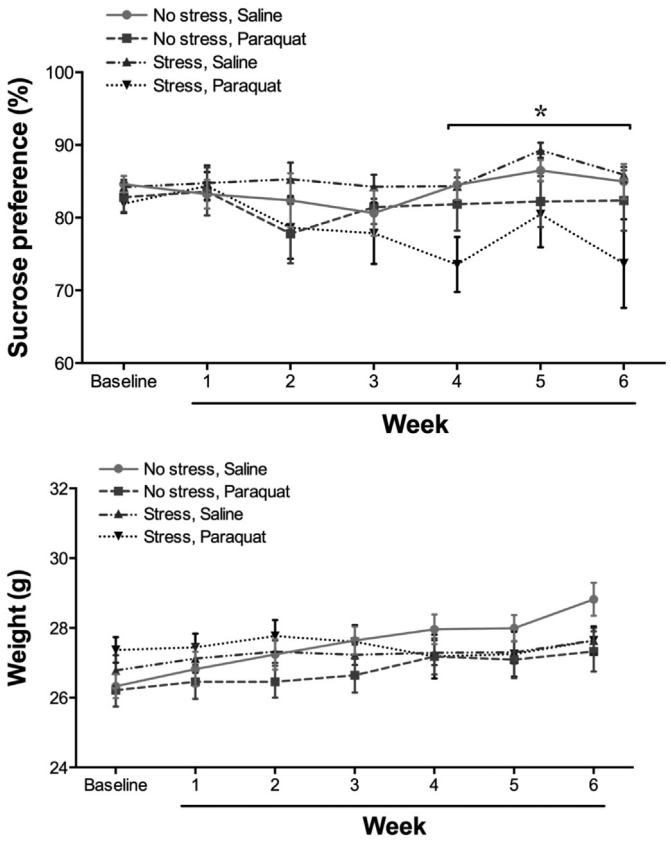

Fig. 5. The top and bottom line graphs depict sucrose preference and weight, respectively, across time as a function of paraquat treatment and chronic intermittent stress. As shown in the top panel, paraquat treated animals had decreased sucrose preference beginning at Week 4; this effect was clearly most prominent in animals receiving concomitant stressor exposure (see text for additional details). The bottom graph shows the diminished weight evident at Weeks 4–6 in paraquat or stressor treated mice, relative to the non-stressed saline treated mice. $^{*}p < 0.05$ relative to saline-treated animals.

reduction, however, the paraquat + stress treatment promoted a modest but statistically significant 9% reduction in sucrose preference at Week 6 relative to baseline.

As shown in Fig. 5 (bottom), mice receiving either paraquat or the stressor gained slightly but significantly less weight overall than saline-treated controls. Specifically, there were significant Paraquat × Time and Stress × Time interactions ($Fs_{5,\ 230} = 4.58$ and 11.83 respectively, $p < 0.01$). Parallelling the time-dependent sucrose effects, the comparisons revealed diminished weight at Weeks 4–6 in paraquat or stressor treated mice ($p < 0.05$). However, in contrast to the sucrose variations, no further weight loss was noted among animals in the paraquat-plus-stressor condition, relative to either of the treatments administered alone. Additionally, neither of the treatments was observed to provoke any overt sickness behaviour (piloerection, lethargy, ptosis).

4. Discussion

Not only does co-morbid depressive illness pose a major threat to quality of life among PD patients, there is evidence to suggest that depression can actually influence the severity and clinical management of PD motor symptoms – and perhaps even affect the progression of the underlying neurodegenerative process (Backer, 2000; Smith et al., 2002; Metz et al., 2005; Kibel and Drenjancević-Perić, 2008; Pålhagen et al., 2008; Smith et al., 2008; Hemmerle et al., 2012; van Dijk et al., 2013). The current findings are consistent with a growing number of reports suggesting that environmental toxicants, and pesticides in particular, may play a role in the development of not only motor impairment, but also non-motor symptoms that are often evident in PD (McDowell and Chesselet, 2012; Freire and Koifman, 2012). Indeed, in the case of paraquat alone, a number of groups have found evidence of both cognitive and anxiety-like symptoms in infrahuman chronic exposure models, and it appears likely that oxidative, inflammatory and potentially even neuroplastic changes occurring in the hippocampus and, to a lesser extent the prefrontal cortex, are implicated in this regard (Litteljohn et al., 2009; Chen et al., 2010; Czerniczyniec et al., 2011; Mangano et al., 2011; Mitra et al., 2011; Songin et al., 2011; Desplats et al., 2012).

Although scant data are available regarding the potential emotional or affective behavioural effects of paraquat, a very recent study found that, in rats, chronic low-dose exposure to paraquat (delivered via osmotic minipumps) induced behavioural signs of learned helplessness in a forced swim test (Campos et al., 2013). Yet, it should be underscored that the Campos et al. (2013) study did not uncover any signs of anhedonia (or cognitive dysfunction for that matter) among the paraquat-treated rats. Seeing as how in the present study the paraquat-induced sucrose preference deficits were only observed in the CIS co-treated animals, it may be the case that exposure to paraquat alone is insufficient to cause such deficits, and that another overlapping insult, such as intermittent exposure to psychologically-relevant stress, is needed to fully "unmask" any underlying neurobehavioural deficit.

The fact that animals receiving the paraquat or stressor treatments gained significantly less weight over time compared to control mice raises the possibility that the sucrose deficits might be related to generalized toxicity or non-specific stressor effects. Yet, it is important to note that, unlike the weight loss, the sucrose reduction was only evident in mice that received the combination of stressor + paraquat treatments. It also warrants reiterating that the sucrose preference test data were collected between 24 and 48 h after pesticide and/or stressor application (i.e., on the "recovery day"), thus limiting any potential confounds related to the possible acute "toxic" effects of either of the treatments. Of course, it should be noted that the observed effects were modest and the fact that the stressor alone had little effect on sucrose is an important caveat in this study. Indeed, we are not able to say that the stressor and paraquat treatment really had any synergistic or additive effects, but rather simply that the combination was required to observe any significant effect at all.

Of course, the obvious question then becomes: "What are the biological mechanisms responsible for this behavioural phenomenon?" In the present investigation we examined four distinct but not mutually exclusive possibilities, namely treatment-induced alterations of: 1) HPA activity (i.e., the classical "stress response"), 2) concentrations and network activity of circulating cytokines (i.e., the peripheral "immunological response"), 3) dopaminergic neurotransmission at the nucleus accumbens (i.e., mesolimbic reward circuitry), and 4) striatal DA activity (i.e., nigrostriatal motor pathway).

. It will be recalled that only in the paraquat-plus-stressor group was striatal DA utilization significantly increased. It is thus tempting to speculate that the combination-treated mice were, in fact, hit "harder" than the paraquat alone-treated animals, and a prospective "compensatory response" (Kuter et al., 2007; Biju and de la Fuente-Fernández, 2009) proved sufficient to stave off striatal DA depletion. While much evidence points to the critical involvement of nucleus accumbens DA dysfunction in the development of anhedonic-like symptoms (Remy et al., 2005), there's certainly precedent for suggesting that alterations of striatal

(caudate/putamen) DA signalling could also play a role. For instance Tadaiesky et al. (2008), reported that striatal and PFC alterations of DA, as well as 5-HT and NE, are the likely cause of emotional and cognitive disturbances induced by 6-OHDA administration in rats. These findings were largely recapitulated in a 2010 study by Santiago and colleagues, which also implicated deficits in hippocampal 5-HT signalling. While not explicitly addressed in the present investigation, findings from our laboratory have indicated that paraquat also influence monoamine activity (5-HT, NE and DA) in the hippocampus and prefrontal cortex (reviewed in Litteljohn et al., 2011b).

Recent work has established a connection between circulating pro-inflammatory cytokines and depressive-like symptoms in PD patients (Menza et al., 2010; Lindqvist et al., 2012). In the present study, however, neither paraquat nor the CIS treatment provoked many significant changes in absolute cytokine concentrations. Yet, the pesticide did increase circulating levels of the haematopoietic colony-stimulating factor, GM-CSF. Since we and others have already demonstrated the capacity of this trophic cytokine to protect against the neurodegeneration caused by paraquat and other environmental insults (Nakagawa et al., 2006; Huang et al., 2007; Choudhury et al., 2011; Mangano et al., 2011), it seems reasonable to suggest that the presently observed GM-CSF increase represents a compensatory homoeostatic response to xenobiotic challenge.

Despite the paucity of cytokine changes, we did find that paraquat imposed a distinct “rigidity” on the circulating cytokine network that was abolished by co-administration of the stressor regimen. Particularly intriguing were the positive correlations between pro-inflammatory cytokines (IFN-γ, TNF-α, IL-17, MIP-1α) and the pro-trophic cytokine, GM-CSF, and the anti-inflammatory cytokine, IL-10. In effect, our data hint at a heightened coordination of peripheral cytokine network action among mice exposed only to paraquat, and a capacity of psychological stress to disrupt such network responding. The task of assigning an overall ‘valence’ to the observed cytokine changes is not straightforward (Litteljohn and Hayley, 2012). Nonetheless, it is our contention that increasing the rigidity of the cytokine network likely constitutes an adaptive response to xenobiotic insult, serving to better position the organism to combat further challenges.

Our findings indicate that paraquat affects HPA activity, peripheral cytokines, and mesolimbic and nigrostriatal DA turnover, some of which might be relevant for the development of depressive-like behaviours. Moreover, it appears that psychological stressors are capable of modulating some of the brain and behaviour effects of paraquat. These data could have implications for how chronic stress is perceived and managed in a neurological context or among individuals with a known history of environmental toxicant exposure.

Conflicts of interest

All authors declare no conflicts of interest.

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