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Sub-chronic exposure to the insecticide dimethoate induces a proinflammatory status and enhances the neuroinflammatory response to bacterial lypopolysaccharide in the hippocampus and striatum of male mice

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ABSTRACT

Dimethoate is an organophosphorus insecticide extensively used in horticulture. Previous studies have shown that the administration of dimethoate to male rats, at a very low dose and during a sub-chronic period, increases the oxidation of lipids and proteins, reduces the levels of antioxidants and impairs mitochondrial function in various brain regions. In this study, we have assessed in C57Bl/6 adult male mice, whether sub-chronic (5 weeks) intoxication with a low dose of dimethoate (1.4 mg/kg) affects the expression of inflammatory molecules and the reactivity of microglia in the hippocampus and striatum under basal conditions and after an immune challenge caused by the systemic administration of lipopolysaccharide. Dimethoate increased mRNA levels of tumor necrosis factor α (TNFα) and interleukin (IL) 6 in the hippocampus, and increased the proportion of Iba1 immunoreactive cells with reactive phenotype in dentate gyrus and striatum. Lipopolysaccharide caused a significant increase in the mRNA levels of IL1β, TNFα, IL6 and interferon-γ-inducible protein 10, and a significant increase in the proportion of microglia with reactive phenotype in the hippocampus and the striatum. Some of the effects of lipopolysaccharide (proportion of Iba1 immunoreactive cells with reactive phenotype and IL6 mRNA levels) were amplified in the animals treated with dimethoate, but only in the striatum. These findings indicate that a sub-chronic period of administration of a low dose of dimethoate, comparable to the levels of the pesticide present as residues in food, causes a proinflammatory status in the brain and enhances the neuroinflammatory response to the lipopolysaccharide challenge with regional specificity.

Introduction

In the last decades, several epidemiological studies have demonstrated a link between exposure to environmental pollutants and the incidence of neurodegenerative disorders (Barlow et al., 2005; Le Couteur et al., 1999; Patel et al., 2006). Among environmental pollutants, agrochemicals are continuously used on a massive scale for global food production and persist as residues in food of both vegetal and animal origin, as well as in air and water (Bolognesi and Morasso, 2000). The evidence that many of these chemicals may be toxic in vivo at extremely low doses, suggest that permitted residue levels in food may be too high (Kapka-Skrzypczak et al., 2011; McKinlay et al., 2008).

Dimethoate (DMT) is an organophosphorus (OP) insecticide of systemic action, extensively used in horticulture for pest treatment in onions, tomatoes, and citric fruits and as an acaricide for treating gardens, vineyards, and field crops (CASAFE, 2007). Previous studies have shown that the administration of DMT to male Wistar rats, at a very low dose and during a sub-chronic period, increases the oxidation of lipids and proteins, reduces the levels of antioxidants and impairs mitochondrial function in various brain regions (Astiz et al., 2009a,b).

Although OP pesticides are known to inhibit acetylcholinesterase activity in the central (CNS) and the peripheral nervous system (Banks and Lein, 2012; Kwong, 2002), a number of observations suggest that OP neurotoxicity is not entirely due to perturbations of cholinergic systems, especially during chronic exposure (Duysen et al., 2001; Kamel and Hoppin, 2004; Rohlman et al., 2011; Zurich et al., 2004). Thus, recent studies suggest that OPs may cause disruption of a number of metabolic and cell signaling pathways that affect cell proliferation, differentiation and survival (Hargreaves, 2012; Rush et al., 2010). Furthermore, there is evidence that acute and chronic OP intoxications are associated with modifications in the basal inflammatory status and in the immune response to an inflammatory challenge (Banks and Lein, 2012; Hirani et al., 2007; Rodgers and Xiong, 1997; Sing and Jiang, 2003). The actions of OPs on the immune system are highly relevant for the brain since an altered neuroinflammatory response under pathological conditions may

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enhance neurodegenerative damage. Indeed, systemic infection may interact with environmental insults to induce exaggerated neuroinflammatory, neurodegenerative and behavioral changes and may have deleterious consequences for the brain when encountered in the context of concomitant chemical toxin, traumatic head injury, or psychological stressor exposure (Diz-Chaves et al., 2012, 2013; Litteljohn et al., 2011; Mangano and Hayley, 2009). We propose that environmental toxins might promote a sensitization of neural tissue that may enhance the neuroinflammatory response to a secondary immune challenge.

Thus, in this study, we have administered to male mice a low dose of DMT which is comparable to the levels of the pesticide present as residues in food. The dose was estimated on the basis of the Maximum Residue Levels (MRLs) established by European Union (http://ec.europa.eu/index_en.htm). We assessed whether sub-chronic intoxication affects inflammatory markers in the hippocampus and striatum under basal conditions and after an immune challenge caused by the systemic administration of lipopolysaccharide (LPS). Systemic administration of LPS has been used in this study since it is known that this treatment alters the levels of different inflammatory molecules in the brain (Dantzer, 2004; Kubera et al., 2011; Monje et al., 2003) and induces the activation of microglia (Tapia-Gonzalez et al., 2008).

Microglia participate in the local inflammatory response of the CNS, releasing a variety of inflammatory mediators, including cytokines such as TNF-α, IL1β and IL6, and chemokines such as interferon γ-inducible protein 10 (IP10; CXCL10) (Binukumar et al., 2011; Nakamura, 2002). In this study we have assessed the effect of DMT exposure on the expression of inflammatory molecules produced by microglia: TNF-α, IL1β, IL6 and IP10. In addition, we assessed immunoreactivity for Iba-1, a marker of microglia since changes in the morphology and/or number of these cells is indicative of an altered neuroinflammatory status.

The hippocampus and the striatum were selected for this study since intoxications with DMT are known to produce alterations in functions controlled by these structures, such as associative learning, associative memory and motor performance (Valenzuela-Harrington et al., 2012). In addition, the hippocampus and the striatum are extremely sensitive to inflammation and oxidative damage (Cerbai et al., 2012) and alterations in these brain structures are associated with important cognitive, affective and neurological disorders in humans (Karen et al., 2001; McDaniel and Moser, 2004; Ramos et al., 2006).

Methods

Chemicals

DMT was obtained as a gift from INTA (Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina) and was of analytical grade. The active ingredient was dissolved at 40% (p/v) in aqueous solution of polyethyleneglycol-400 at 25%.

Animals and treatments

Animals were handled in accordance with the guidelines presented in the UFAW Handbook on the Care and Management of Laboratory Animals and following the European Union guidelines (Council Directives 86/609/EEC and 2010/63/UE). Experimental procedures were approved by our institutional animal use and care committee. Special care was taken to minimize suffering and to reduce the number of animals used to the minimum required for statistical accuracy. C57BL/6 male mice (7 weeks old) weighing 23 ± 1.1 g were purchased to Harlan Laboratories. Upon arrival, mice were allowed to acclimatize for 10 days before starting the experiment, they were maintained under controlled conditions of temperature (25 ± 2 °C), and a normal photoperiod of 12 h dark and 12 h light, and fed with standard chow and water ad libitum. Body weight evaluation was performed every week during the experiment. Animals were randomly divided into two groups of twelve mice each, assigned as (i), vehicle group (injected i.p. with 25% polyethylene-glycol-400) and (ii), DMT-treated group (injected i.p. with 1.4 mg DMT/kg body weight dissolved in 25% polyethylene-glycol-400). All animals were injected three times a week for 5 weeks. The selection of the DMT dose was based on two known parameters. We first considered the established lethal dose 50 of DMT. The selected dose (1.4 mg/kg) is a 0.9% of LD50 which is considered low. In addition, we estimated the dose on the basis of the Maximum Residue Levels (MRLs) established by European Union (http://ec.europa.eu/index_en.htm). The selected dose of DMT for the present study is comparable with the doses used by other researchers in studies with similar treatment schedules (Ayed-Boussema et al., 2012; Farag et al., 2007).

Twenty-four hours before the end of the treatment with vehicle or DMT, each group of mice was divided in two. One of the subgroups received an i.p. injection of vehicle (phosphate buffered saline, PBS) and the other an i.p. injection of 5 mg/kg bw of lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4, L2630 Sigma-Aldrich Corporation, St Louis, MO, USA) dissolved in PBS. Animals were killed 24 h after LPS or PBS administration. Therefore, four groups of animals were generated: a control group treated with vehicles for pesticide and for LPS (VEH/VEH, n = 6), a group treated with DMT and the vehicle for LPS (DMT/VEH, n = 6), a group treated with the vehicle for DMT and LPS (VEH/LPS, n = 6) and a group treated with DMT and LPS (DMT/LPS, n = 6). The dose of LPS was based on previous studies (Diz-Chaves et al., 2012, 2013; Qin et al., 2007).

Sample collection

Animals were killed by decapitation and the brains were quickly removed. The right hemispheres were immersed in 4% paraformaldehyde (Sigma-Aldrich Corporation) in 0.1 M phosphate buffer, pH 7.4 during 48 h and then rinsed with phosphate buffer and stored at −20 °C in a cryoprotective solution. From the left hemispheres the striatum and the hippocampus were dissected and stored at −80 °C.

Quantitative real-time polymerase chain reaction

Interleukin 1β (IL1β), tumor necrosis factor-α (TNF-α), interleukin (IL) 6 and interferon-γ-inducible protein 10 (IP10) mRNA expression levels were assessed in the hippocampus and striatum by quantitative real-time polymerase chain reaction. Tissue was homogenized and RNA was extracted using TRI reagent® solution (Ambion, USA). First strand cDNA was prepared from 2 μg RNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. After reverse transcription, cDNA was diluted 1:4 and 1:8 for the target genes and 1:2000 for the endogenous control (18S). Five μL of these cDNA solutions were amplified by real-time PCR in a 15 μL volume reaction using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using the ABI Prism 7500 Sequence Detection System (Applied Biosystems) with conventional Applied Biosystems cycling parameters (40 cycles of changing temperatures, first at 95 °C for 15 s and then 60 °C for a minute).

All the primer sequences were designed using Primer Express software (Applied Biosystems). IL1β: forward 5′-CGACAAAATACC TGTGGCCT-3′ reverse 5′-TTCTTTGGGTATTGCTTGGG-3′; TNF-α: forward 5′-GAAAAGCAAGCAGGCAACCA-3′ reverse 5′-CGGATCATGCTT TCTGTGCTC-3′; IL6: forward 5′-GAAACCGCTATGAAGTTCCTCTCTG-3′ reverse 5′-TGTTGGGAGTGGTATCCTCTGTGA-3′ and IP10: forward 5′-CAGGAGAATGAGGGCCATAGG-3′ reverse 5′-CGGATTCAGACATCT CTGCTCAT-3′. IL1β, TNF-α, IL6 and IP10 gene expressions were normalized to 18S as endogenous control.

Immunohistochemistry

Sagittal sections of the brain, 50 μm thick, were obtained using a Vibratome (VT 1000S, Leica Microsystems, Wetzlar, Germany). Immunohistochemistry was carried out in free-floating sections under moderate shaking. Endogenous peroxidase activity was quenched for 15 min at room temperature in a solution of 3% hydrogen peroxide in 50% methanol. After several washes in 0.1 M phosphate buffer (pH 7.4), containing 0.3% BSA and 0.3% Triton X-100 (washing buffer), sections were incubated overnight at 4 °C with a rabbit polyclonal antibody against the microglia marker Iba1 (Ionized calcium binding adaptor molecule 1) corresponding to C-terminus (Wako Chemical Industries, Japan; diluted 1:2000). Primary antibody was diluted in washing buffer containing 5% normal goat serum. After incubation with the primary antibody, sections were rinsed in washing buffer and incubated for 2 h at room temperature with biotinylated goat anti-rabbit immunoglobulin G (Pierce Antibody; Rockford, IL 61101, USA; diluted 1:300 in washing buffer). After several washes in buffer, sections were incubated for 90 min at room temperature with avidin–biotin peroxidase complex (ImmunoPure ABC peroxidase staining kit, Pierce). The reaction product was revealed by incubating the sections with 2 μg/ml 3,3′-diaminobenzidine (Sigma-Aldrich Corporation) and 0.01% hydrogen peroxide in 0.1 M phosphate buffer. Then, sections were dehydrated, mounted on gelatinized slides and examined with a Leitz Laborlux microscope.

Morphometric analysis

The morphometric analysis was performed by an investigator that was unaware of the identity of the experimental groups. The number of Iba1-immunoreactive cells in the hilus of dentate gyrus and in the striatum (caudate putamen) was estimated with the optical disector method, using total section thickness for disector height at 40× and a counting frame of 220 × 220 μm. Section thickness was measured using a digital length gauge device (Heidenhain-Metro MT 12/ND221; Traunreut, Germany) attached to the stage of a Leitz microscope. A total of 30 counting frames were assessed per animal.

The quantity of Iba1 immunoreactive cells with different morphologies was also assessed. Ninety cells were analyzed for each animal in the hilus of the dentate gyrus and in the striatum in sections of the right hemisphere located between 1.20 and 1.80 mm from the midline, according to the atlas of Paxinos and Franklin (2001). Iba1-immunoreactive cells with few cellular processes (two or less), cells showing four short branches and also cells with numerous cell processes and a small cell body were classified as resting microglia (Diz-Chaves et al., 2012, 2013). Iba1-immunoreactive cells with large somas and retracted and thicker processes and cells with amoeboid cell body, numerous short processes and intense Iba1 immunostaining, were classified as reactive microglia (Diz-Chaves et al., 2012, 2013).

Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics 1.9 software (Chicago, IL, USA). We first assessed the normal distribution of data by Kolmogorov–Smirnov's test and homoscedasticity by Levene's test. Data were then analyzed with a three-factor ANOVA, considering DMT treatment, LPS treatment and brain region as fixed factors. For post hoc analysis the Bonferroni's test was used. Differences were considered statistically significant at $p \leq 0.05$. Data are presented as mean ± SEM.

Results

Effect of DMT treatment on the mRNA levels of IL1β in the hippocampus and striatum under basal conditions and after LPS administration

The mRNA levels of IL1β were assessed in the hippocampus and the striatum (Figs. 1A and B, respectively). Data were analyzed using a three-factor ANOVA, with DMT, LPS and brain region as fixed factors. Three-factor ANOVA revealed a significant interaction between DMT and brain region [$F_{(1, 37)} = 6.356$, $p = 0.016$] and between LPS and brain region [$F_{(1, 37)} = 9.029$, $p = 0.005$]. In contrast, no significant interaction between DMT and LPS was detected. DMT did not significantly affect IL1β mRNA levels. In contrast, LPS showed a significant effect on IL1β mRNA levels [$F_{(1, 37)} = 107.945$, $p < 0.001$]. In addition there was a significant effect of brain region [$F_{(1, 37)} = 13.822$, $p = 0.001$].

The post-hoc analysis showed a significant increase in IL1β mRNA levels after LPS administration in both the hippocampus (VEH/LPS vs VEH/VEH, $p = 0.003$) and the striatum (VEH/LPS vs VEH/VEH, $p = 0.016$). The effect of LPS was significantly higher in the striatum than in the hippocampus (VEH/LPS in hippocampus vs VEH/LPS in the striatum, $p = 0.020$). DMT did not significantly affect the response to LPS (no significant differences between VEH/LPS and DMT/LPS groups). The treatment with DMT in combination with LPS increased IL1β mRNA expression versus basal conditions in striatum (DMT/LPS vs VEH/VEH, $p = 0.008$) and the effect was significantly higher, but not statistically significant, in the striatum than in the hippocampus (DMT/LPS in hippocampus vs DMT/LPS in the striatum, $p = 0.061$). These results indicate that DMT did not significantly affect IL1β mRNA levels in the hippocampus and the striatum under basal conditions

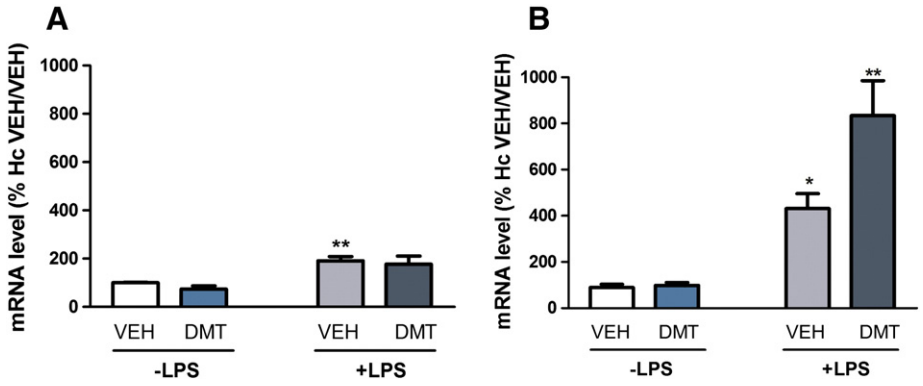

Fig. 1. Effect of DMT treatment on the mRNA levels of IL1β in the hippocampus (A) and striatum (B) under basal conditions and after LPS administration. VEH, animals treated 5 weeks with pesticide vehicle (25% polyethylene-glycol-400). DMT, animals treated 5 weeks with 1.4 mg/kg bw DMT. VEH and DMT groups were injected with PBS (−LPS) or 5 mg/kg bw LPS (+LPS) and killed 24 h later. The results are expressed as mean + SEM. *,** Significant differences (*$p < 0.05$, **$p < 0.01$) compared to VEH/VEH (control animals).

and did not significantly modify the response of IL1β mRNA levels to LPS.

Effect of DMT treatment on the mRNA levels of TNFα in the hippocampus and striatum under basal conditions and after LPS administration

The mRNA levels of TNFα were assessed in the hippocampus and the striatum (Figs. 2A and B, respectively). Three-factor ANOVA revealed a significant interaction between DMT, LPS and brain region [$F_{(1, 37)} = 10.389$, $p = 0.003$]. However, no significant interactions were detected between DMT and LPS, DMT and brain region or LPS and brain region. There was a significant effect of DMT [$F_{(1, 37)} = 16.602$, $p < 0.001$] a significant effect of LPS [$F_{(1, 37)} = 228.935$, $p < 0.001$] and a significant effect of the brain region [$F_{(1, 37)} = 52.549$, $p < 0.001$].

The post-hoc analysis revealed a significant effect of DMT, increasing mRNA levels of TNFα in the hippocampus (DMT/VEH vs VEH/VEH, $p = 0.002$). In contrast, DMT did not significantly affect TNFα mRNA levels in the striatum. Therefore, the mRNA levels of TNFα were significantly higher in the hippocampus than in the striatum in DMT treated animals ($p < 0.001$). LPS induced a significant increase in TNFα mRNA levels in the hippocampus and striatum (VEH/LPS vs VEH/VEH, $p < 0.001$ for both regions). The effect of LPS was stronger in the hippocampus. Therefore, TNFα mRNA levels were significantly higher in the hippocampus than in the striatum of LPS treated animals ($p = 0.001$). The effect of LPS was not significantly affected by the treatment with DMT. The treatment with DMT in combination with LPS increased TNFα mRNA expression versus basal conditions (DMT/LPS vs VEH/VEH, $p < 0.001$ in both regions) and the effect was significantly higher, but not statistically significant, in the hippocampus than in the striatum (DMT/LPS in hippocampus vs DMT/LPS in the striatum, $p = 0.053$). These results indicate that, under basal conditions, DMT increases the mRNA levels of TNFα in the hippocampus but not in the striatum and that DMT did not significantly modify the response of TNFα mRNA levels to LPS.

Effect of DMT treatment on the mRNA levels of IL6 in the hippocampus and striatum under basal conditions and after LPS administration

The mRNA levels of IL6 were assessed in the hippocampus and the striatum (Figs. 3A and B, respectively). Three-factor ANOVA revealed a significant interaction between LPS and brain region [$F_{(1, 36)} = 4.876$, $p = 0.034$] a significant effect of DMT [$F_{(1, 36)} = 75.634$, $p < 0.001$] and a significant effect of LPS [$F_{(1, 36)} = 316.032$, $p < 0.001$].

The post-hoc analysis revealed a significant effect of DMT, increasing IL6 mRNA levels in the hippocampus (DMT/VEH vs VEH/VEH, $p = 0.001$) but not in the striatum. However, the levels of IL6 mRNA were not significantly different between the hippocampus and striatum of DMT treated animals. LPS treatment increased IL6 mRNA levels in the hippocampus (VEH/LPS vs VEH/VEH, $p = 0.001$) and the striatum (VEH/LPS vs VEH/VEH, $p = 0.003$). The combined treatment with DMT and LPS increase IL6 mRNA levels compared to vehicle-treated animals (DMT/LPS vs VEH/VEH, $p < 0.001$ for both regions). DMT treatment did not significantly modify the effect of LPS on IL6 mRNA levels in the hippocampus. However, DMT increased the effect of LPS on IL6 mRNA levels in the striatum (DMT/LPS vs VEH/LPS, $p = 0.007$). These results reveal an effect of DMT, increasing the mRNA levels of IL6 in the hippocampus under basal conditions and enhancing the response of IL6 mRNA levels to LPS in the striatum.

Effect of DMT treatment on the mRNA levels of IP10 in the hippocampus and striatum under basal conditions and after LPS administration

The mRNA levels of IP10 were assessed in the hippocampus and the striatum (Figs. 4A and B respectively). Three-factor ANOVA did not reveal a significant interaction between DMT and LPS or between the treatments and the two brain regions analyzed. LPS had a significant effect on IP10 mRNA levels [$F_{(1, 36)} = 125.820$, $p < 0.001$].

The post-hoc analysis revealed a significant effect of LPS in the hippocampus (VEH/LPS vs VEH/VEH, $p = 0.001$) but not in the striatum. The combined treatment with DMT and LPS increased also IP10 mRNA expression levels compared to vehicle-treated animals (DMT/LPS vs VEH/VEH in hippocampus $p = 0.003$ and in striatum $p = 0.012$). However, DMT did not modify the effect of LPS. These findings indicate that DMT does not affect IP10 mRNA levels, neither under basal conditions nor after LPS treatment.

Effects of DMT treatment on the number of Iba1 immunoreactive cells in the hilus of the dentate gyrus and the striatum under basal conditions and after LPS administration

Iba1 immunoreactivity was evaluated in the hilus of the dentate gyrus and the striatum (Figs. 5 and 6). In a first analysis we evaluated the total number of Iba1 immunoreactive cells in these structures (Figs. 7A and B). Data was analyzed by three-factor ANOVA, using treatment with DMT, treatment with LPS and brain region as fixed factors. ANOVA revealed a significant interaction between DMT, LPS and brain region [$F_{(1,40)} = 9.520$, $p = 0.004$], a significant interaction between DMT and LPS [$F_{(1, 40)} = 7.086$, $p = 0.011$], a significant interaction between LPS and brain region [$F_{(1, 40)} = 4.744$, $p = 0.035$], a significant effect of LPS [$F_{(1, 40)} = 182.024$, $p < 0.001$] and a significant effect of the brain region [$F_{(1, 40)} = 130.750$, $p < 0.001$].

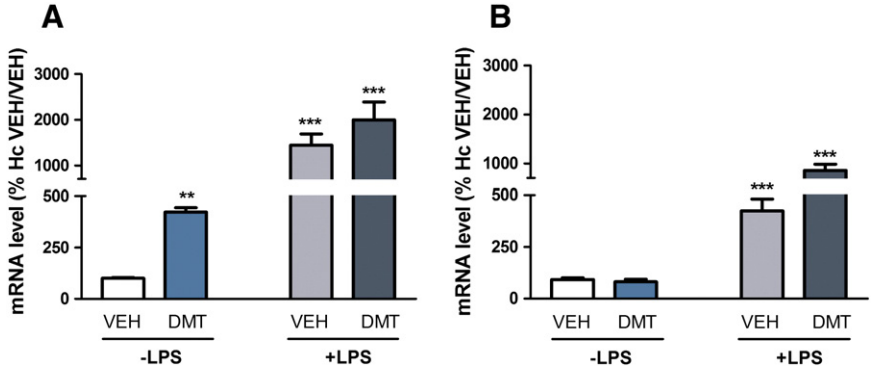

Fig. 2. Effect of DMT treatment on the mRNA levels of TNFα in the hippocampus (A) and striatum (B) under basal conditions and after LPS administration. VEH, animals treated 5 weeks with pesticide vehicle (25% polyethylene-glycol-400). DMT, animals treated 5 weeks with 1.4 mg/kg bw DMT. VEH and DMT groups were injected with PBS (−LPS) or 5 mg/kg bw LPS (+LPS) and killed 24 h later. The results are expressed as mean + SEM. **,*** Significant differences (**$p < 0.01$, ***$p < 0.001$) compared to VEH/VEH (control animals).

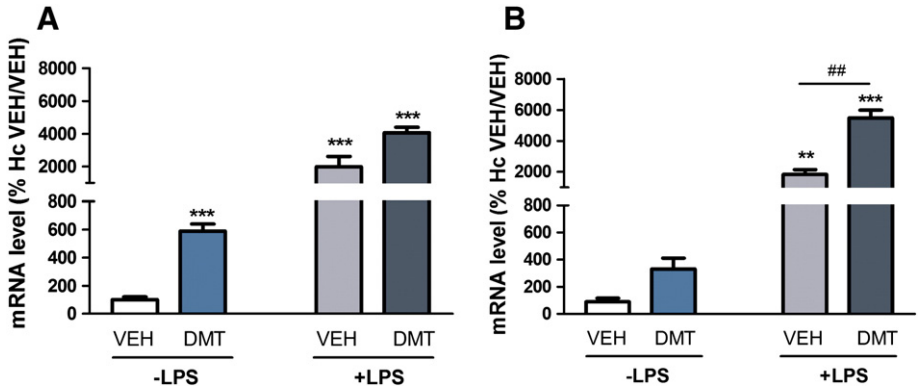

Fig. 3. Effect of DMT treatment on the mRNA levels of IL6 in the hippocampus (A) and striatum (B) under basal conditions and after LPS administration. VEH, animals treated 5 weeks with pesticide vehicle (25% polyethylene-glycol-400). DMT, animals treated 5 weeks with 1.4 mg/kg bw DMT. VEH and DMT groups were injected with PBS (−LPS) or 5 mg/kg bw LPS (+LPS) and killed 24 h later. The results are expressed as mean + SEM. **,*** Significant differences (**$p < 0.01$, ***$p < 0.001$) compared to VEH/VEH (control animals). ## Significant differences ($p < 0.01$) between the groups identified by the bar.

The post-hoc analysis revealed that the striatum has a significantly higher number of Iba-1 immunoreactive cells than the hilus of the dentate gyrus under basal conditions (VEH/VEH group; $p < 0.001$), after DMT treatment ($p = 0.006$) and after the treatment with DMT and LPS ($p = 0.002$). However, the animals treated only with LPS (VEH/LPS) showed a similar number of Iba-1 immunoreactive cells in both structures. This is because the treatment of LPS resulted in a significant increase in the number of Iba-1 immunoreactive cells in the hilus of the dentate gyrus ($p = 0.002$) but not in the striatum. Interestingly, LPS significantly increased the number of Iba-1 immunoreactive cells in the striatum of the animals treated with DMT ($p < 0.001$). This resulted in a significant difference in the number of Iba-1 immunoreactive cells in the striatum between the animals treated with LPS alone and the animals treated with DMT and LPS ($p = 0.011$). In contrast, LPS has the same effect in the hilus of the dentate gyrus, independently of whether the animals were treated with DMT or not. In addition, DMT did not significantly affect the number of Iba-1 immunoreactive cells in the animals not stimulated with LPS.

Effects of DMT treatment on the proportion of Iba1 immunoreactive cells with reactive phenotype in the hilus of the dentate gyrus and the striatum under basal conditions and after LPS administration

Iba-1 immunoreactive cells were classified according to their morphology as described previously (Diz-Chaves et al., 2012, 2013). Those cells that showed large somas and retracted and thicker processes and cells showing amoeboid cell body with numerous short processes and intense Iba1 immunostaining, were classified as reactive (Figs. 5 and 6). The proportion of Iba-1 immunoreactive cells with reactive phenotype in the hilus of the dentate gyrus and in the striatum is shown in Figs. 7C and D, respectively. ANOVA revealed a significant interaction between DMT and LPS [$F_{(1, 36)} = 10.880$, $p = 0.002$], a significant interaction between LPS and brain region [$F_{(1, 36)} = 5.072$, $p = 0.031$], a significant effect of DMT [$F_{(1, 36)} = 26.246$, $p < 0.001$], a significant effect of LPS [$F_{(1, 36)} = 261.936$, $p < 0.001$] and a significant effect of brain region [$F_{(1, 36)} = 14.313$, $p = 0.001$].

The post-hoc analysis revealed that the treatment with DMT (DMT/VEH) increased the proportion of Iba-1 immunoreactive cells with reactive phenotype in both the hilus of the dentate gyrus and the striatum ($p = 0.001$ and $p = 0.025$, respectively) versus animals treated with vehicle (VEH/VEH). The hilus of the dentate gyrus showed a higher response to DMT than the striatum in this parameter ($p = 0.014$). LPS resulted in a significant increase in the number of Iba-1 immunoreactive cells with reactive phenotype in the hilus of the dentate gyrus and the striatum ($p = 0.001$ versus the VEH/VEH group in both structures). The combined treatment with DMT and LPS increased the ratio of Iba-1 immunoreactive cells with reactive phenotype in both regions compared with vehicle-treated animals (DMT/LPS vs VEH/VEH in hippocampus $p < 0.001$ and in striatum $p = 0.002$). However, the effect of LPS was not significantly modified by the treatment with DMT.

Discussion

In this study we have assessed the effects of a sub-chronic period of administration of a low dose of the OP insecticide DMT on neuroinflammation parameters in male mice. The dose of DMT used in the present study is comparable to those found as residues

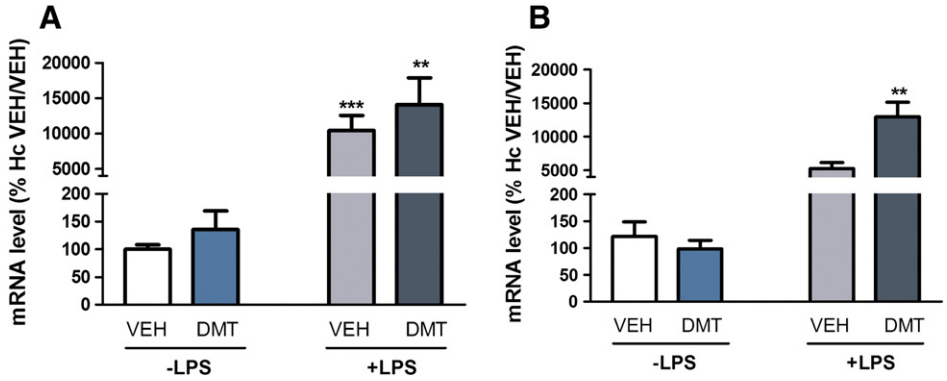

Fig. 4. Effect of DMT treatment on the mRNA levels of IP10 in the hippocampus (A) and striatum (B) under basal conditions and after LPS administration. VEH, animals treated 5 weeks with pesticide vehicle (25% polyethylene-glycol-400). DMT, animals treated 5 weeks with 1.4 mg/kg bw DMT. VEH and DMT groups were injected with PBS (−LPS) or 5 mg/kg bw LPS (+LPS) and killed 24 h later. The results are expressed as mean + SEM. **,*** Significant differences (**$p < 0.01$, ***$p < 0.001$) compared to VEH/VEH (control animals).

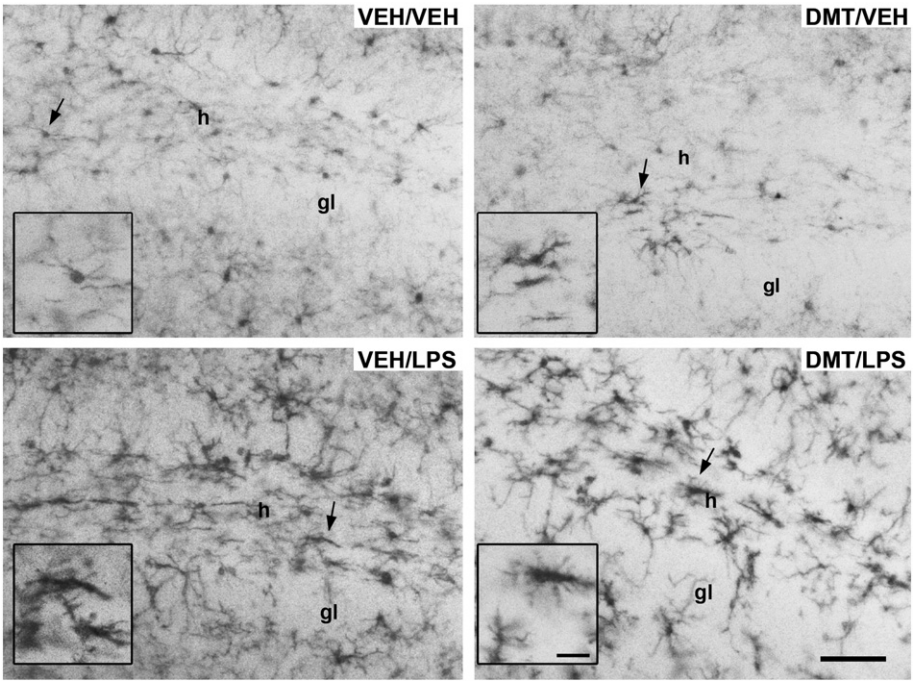

Fig. 5. Representative examples of sections of the dentate gyrus immunostained for Iba1. VEH/VEH, animal treated with vehicles. DMT/VEH, animal treated with DMT. VEH/LPS, animal injected with LPS. DMT/LPS, animal treated with DMT and injected with LPS. Arrows point to Iba1 immunoreactive cells that are shown at high magnification in the insets. h, Hilus of the dentate gyrus; gl, granule cell layer. Scale bar, 60 μm. Scale bar in the insets, 15 μm.

in food and water. As expected by previous studies (Ayed-Boussema et al., 2012; Farag et al., 2007), DMT did not significantly affect body weight in male mice. However, DMT increased mRNA levels of TNFα and IL6 in the hippocampus of male mice. In addition, DMT treatment increased the proportion of Iba1 immunoreactive microglia cells with reactive phenotype in the hilus of the dentate gyrus and in a lesser extent in the striatum. Under basal conditions, microglia displays a ramified morphology and is referred to as "resting" microglia, but upon subtle changes in their micro-environment, or as a consequence of pathological insults, they rapidly transform into an activated reactive state. Reactive microglia show large somas and retracted and thicker processes or an amoeboid cell body and intense Iba1 immunostaining (Diz-Chaves et al., 2012, 2013). In the two brain regions studied, DMT treatment increased the proportion

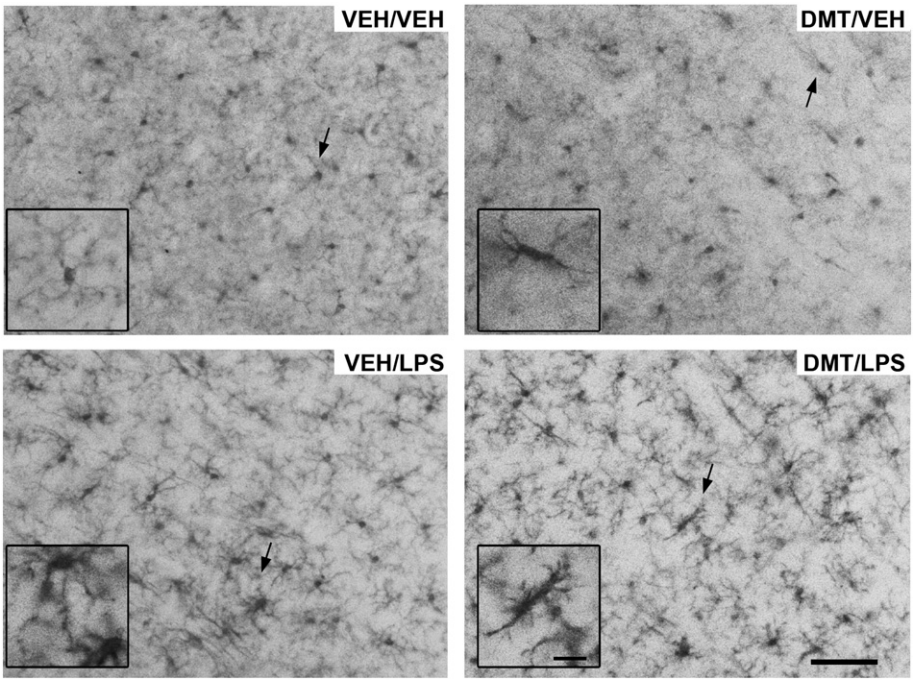

Fig. 6. Representative examples of sections of the striatum immunostained for Iba1. VEH/VEH, animal treated with vehicles. DMT/VEH, animal treated with DMT. VEH/LPS, animal injected with LPS. DMT/LPS, animal treated with DMT and injected with LPS. Arrows point to Iba1 immunoreactive cells that are shown at high magnification in the insets. Scale bar, 60 μm. Scale bar in the insets, 15 μm.

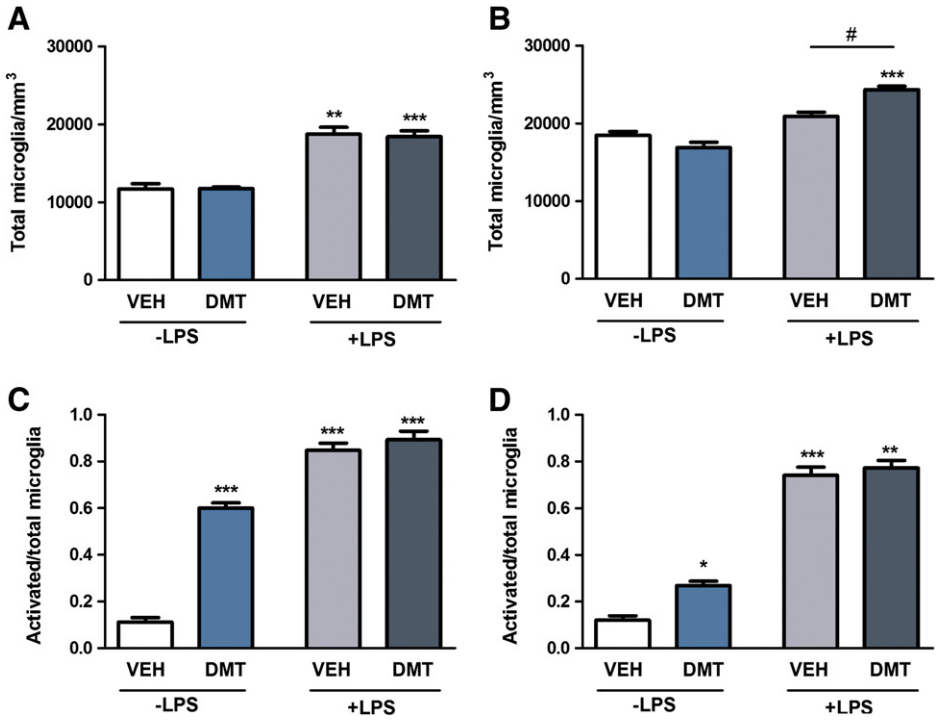

Fig. 7. Number of Iba1 immunoreactive cells/mm^3 and proportion of cells with reactive morphology in the hilus of dentate gyrus of the hippocampus and in the striatum. A and B) Total number of Iba1 immunoreactive cells in the hilus of the dentate gyrus and the striatum, respectively. C and D) Proportion of Iba1 immunoreactive cells with reactive morphology in the hilus of the dentate gyrus and the striatum, respectively. VEH, animals treated 5 weeks with pesticide vehicle (25% polyethylene-glycol-400). DMT, animals treated 5 weeks with 1.4 mg/kg bw DMT. VEH and DMT groups were injected with PBS (−LPS) or 5 mg/kg bw LPS (+LPS) and killed 24 h later. The results are expressed as mean + SEM. *,**,*** Significant differences (*$p < 0.05$, **$p < 0.01$; ***$P < 0.001$) compared to VEH/VEH (control animals). # Significant difference ($P < 0.05$) between the groups identified by the bar.

of Iba1 immunoreactive cells with these morphological characteristics, without affecting the total number of microglia cells. Reactive microglia release proinflammatory molecules, such as IL6 and TNFα (Nakamura, 2002). Therefore, the increase in the mRNA levels of TNFα and IL6 after DMT treatment in the hippocampus is in agreement with the increase in the proportion of microglia cells showing a reactive phenotype in the hilus of the dentate gyrus. However, activated microglia also release IL1β and IP10, which were unaffected by DMT in the hippocampus. Furthermore, the proportion of reactive microglia was slightly increased by DMT in the striatum, while in this brain region DMT did not significantly affect the mRNA levels of any of the inflammatory molecules studied. This suggests that DMT per se does not induce a fully activated microglia phenotype. The anti-inflammatory properties that have been attributed to IL6 (Spooren et al., 2011) may contribute to moderate the inflammatory response of microglia.

The modifications in the expression of inflammatory mediators and in the microglia phenotype suggest that DMT induces a proinflammatory status in the brain. A chronic proinflammatory response, involving long standing activation of microglia and sustained release of inflammatory mediators, may contribute or be the cause of the induction of oxidative stress by DMT (Astiz et al., 2009a,b; Frank-Cannon et al., 2009; Rohlman et al., 2011). In addition, the increase in the number of reactive microglia may contribute to the increase in the production of prostaglandins PGE_2 and $PGF_{2\alpha}$ detected in the brain of animals treated with DMT (Astiz et al., 2012), since the expression of cyclooxygenase-2, a key enzyme responsible for the synthesis of inflammation-related prostaglandins, is upregulated in reactive microglia (Hald and Lotharius, 2005).

An important finding of our study is the differential vulnerability to DMT of the two brain regions studied. DMT treatment resulted in a higher increase in the mRNA levels of TNFα in the hippocampus (4.2 fold) than in the striatum. The proportion of microglia cells with reactive morphology was also higher in the hilus of the dentate gyrus than in the striatum after DMT treatment. The causes for these regional differences in the effect of DMT are unknown. However, differences in the inflammatory response between the hippocampus and the striatum have been previously reported in rats treated with LPS (Bay-Richter et al., 2011; Bossù et al., 2012). Our present findings also show a different immune response of the hippocampus and the striatum to LPS. Thus, LPS significantly increased the number of microglial cells in the hilus of the dentate gyrus but not in the striatum. TNFα mRNA levels increased 14.4 fold in the hippocampus versus 4.5 fold in the striatum. In contrast, LPS induced a more prominent increase in IL1β mRNA levels in the striatum (4.8 fold) than in the hippocampus (1.9 fold). Regional differences in cellular composition and neuron-glia communication may be involved in these differences.

Some of the effects of LPS were enhanced in the animals treated with DMT. Thus, LPS produced a higher increase in IL6 mRNA levels in the striatum of the animals pretreated with DMT compared to the animals pretreated with vehicle. In addition, LPS increased the total number of microglial cells in the striatum, but only in the animals pretreated with DMT. Therefore, the pretreatment with DMT enhances the neuroinflammatory response to LPS.

Further studies should determine the mechanisms involved in the actions of DMT on microglia and on the expression of inflammatory molecules. The best characterized mechanism of action of OP pesticides is the inhibition of acetylcholinesterase activity in the central and peripheral nervous system (Banks and Lein, 2012; Farag et al., 2007; Kwong, 2002). However, previous studies using similar treatments with DMT as the one used in our present experiments did not detect modifications in acetylcholinesterase activity (Ayed-Boussema et al., 2012; Farag et al., 2007). Furthermore, acetylcholine reduces the inflammatory response of glial cells (Darvesh et al., 2010; Lee, 2013; Nizri et al., 2008) and the treatment of microglia

cultures with cholinesterase inhibitors suppresses the LPS-induced release of inflammatory molecules, such as TNFα, IL1β and NO (Hwang et al., 2010). Therefore, the proinflammatory status induced by DMT is not likely to be related to cholinesterase inhibition and accumulation of acetylcholine. Therefore, other metabolic and cell signaling pathways should be explored.

The action of DMT on inflammatory molecules may be mediated by its metabolite omethoate. DMT is biotransformed in the liver microsomes by its oxidation to omethoate (Farag et al., 2007; Hassan et al., 1969), which is able to cross the blood–brain barrier and exerts proinflammatory actions (Wang et al., 2011). Another important point that should be explored is the participation of astrocytes in the action of DMT, since astrocytes interact with microglia and participate in the control of the neuroinflammatory response (Broussard et al., 2012) and there is some evidence that astrocytes are also important for the mechanism of OP-mediated inflammation (Banks and Lein, 2012).

Conclusion

In summary, our findings indicate that even at a very low dose, similar to those present as residues in food, a sub-chronic period of administration of DMT induces a proinflammatory status in the hippocampus (and in a lesser extent in the striatum) and potentiates the neuroinflammatory response of the striatum (and in a lesser extent of the hippocampus) to a subsequent inflammatory challenge. These neuroimmune alterations caused by this insecticide could increase the vulnerability to develop behavioral and cognitive deficits after a continued exposure through years and may be related with the reported effects of DMT on associative learning, memory and motor function (Valenzuela-Harrington et al., 2012).

Conflict of interest statement

The authors declare that they have no competing interests.

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