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The opioid antagonist, β-funaltrexamine, inhibits lipopolysaccharide-induced neuroinflammation and reduces sickness behavior in mice

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ABSTRACT

Brain pathologies such as neurodegenerative diseases, infection, traumatic brain injury, and mood disorders produce enormous personal and economic burdens. It is well established that neuroinflammation plays an important role in the etiology and/or manifestation of such disorders. Previously, we discovered that beta-funaltrexamine (β-FNA) inhibits inflammatory signaling in human astrocytes in vitro, resulting in reduced expression of proinflammatory cytokines/chemokines. The present study examines the effects of peripherally administered β-FNA on lipopolysaccharide (LPS)-induced neuroinflammation and sickness behavior in vivo. Adult male C57BL/6J mice were administered β-FNA and were then immediately administered bacterial lipopolysaccharide (LPS). At 24 h post-injections, sickness behavior was assessed in an open-field test. Following behavioral analysis plasma and brains were collected. Levels of interleukin-6 (IL-6), interferon-γ inducible protein-10 (CXCL10), and monocyte chemoattractant protein-1 (CCL2) were determined by enzyme-linked immunosorbant assay (ELISA). At 24 h post-LPS injection, IL-6, CCL2 and CXCL10 were increased in the plasma, whereas, only CCL2 and CXCL10 were elevated in the brain. β-FNA significantly inhibited LPS-induced CXCL10 and CCL2 expression in brain, but minimally or not at all in the plasma. LPS-induced sickness behavior, as indicated by a reduction in distance moved, was prevented by β-FNA. Overall, CXCL10 expression in the brain was most positively and significantly correlated with sickness behavior; whereas, anxiety-like behavior was most positively and significantly correlated with IL-6 and CCL2 levels in the plasma and levels of CXCL10 and CCL2 in the brain. The reduction in sickness behavior may be in part due to decreased chemokine expression in the brain; further examination of the anti-inflammatory and neuroprotective effects of β-FNA is warranted.

1. Introduction

Neuroinflammation occurs in a wide range of neurological disorders - from central nervous system (CNS) infection and trauma to neurodegenerative diseases and psychiatric disorders [1–5] - yet treatment regimens for these conditions typically do not include an anti-inflammatory drug. Interestingly, emerging evidence suggests that the therapeutic effectiveness of some drugs (i.e., anti-depressants and anti-psychotics) used to treat mood disorders is due in part, to their anti-inflammatory actions [6–9]. Thus, there is an urgent need to identify novel anti-inflammatory agents that readily cross the blood-brain barrier (BBB) as therapeutic options in the treatment of mood and neurological disorders involving neuroinflammation.

Previously we discovered that the *mu*-opioid receptor antagonist, β-funaltrexamine (β-FNA), inhibits inflammatory signaling in human astroglial (A172) cells and in normal human astrocytes [10]. More specifically, in vitro, proinflammatory-induced p38 and NF-κB activation stimulated by tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), or bacterial lipopolysaccharide (LPS)], and downstream chemokine and inducible nitric oxide synthase (iNOS) expression, all are inhibited by β-FNA [11,12]. Further characterization of the anti-inflammatory actions of β-FNA has focused largely on IL-1β-induced expression of the chemokine, interferon-γ inducible protein-10 (CXCL10) [12,13]. Although β-FNA is known to bind opioid receptors, considerable evidence indicates that inhibition of CXCL10 expression by β-FNA is not mediated through the classical *mu* opioid receptor (MOR) or other opioid receptors as the effects of β-FNA on CXCL10 expression were not altered by the general opioid receptor antagonist, naltrexone nor by the MOR selective antagonist, D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH_2 (CTAP) [12,13]. Furthermore, neither the *kappa* opioid receptor selective (nor-binaltorphimine, Nor-BNI) nor the nociception/orphanin FQ receptor (ORL)-selective ([$Nphe^1$]-$nociceptin_{1-13}$ amide, NoCi) antagonists effectively blocked β-FNA effects on pro-inflammatory-

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induced CXCL10 expression [12]. It is also important to note that, unlike β-FNA, none of these opioid receptor antagonists inhibited pro-inflammatory TNFα-induced CXCL10 expression, even at 3-fold higher concentrations. Together, these findings suggest that the anti-inflammatory actions of β-FNA are mediated not via its anti-opiate actions, but by a target or targets yet to be identified. However, outside of our previous report [13], very little is known about the effects of β-FNA on neuroinflammation in vivo. Therefore, the main objective of this study was to determine the extent to which peripherally administered β-FNA could prevent LPS-induced neuroinflammation in C57BL/6J mice. We also wanted to determine whether β-FNA treatment could reduce sickness behavior in LPS-treated mice. Together, insights gained from these studies will be crucial to further investigation of the therapeutic potential of β-FNA and related compounds in the treatment of neurological disorders involving neuroinflammation.

2. Materials and methods

2.1. Animals

Seven-week old male C57BL/6J mice, purchased from Jackson Laboratories (Bar Harbor, ME), were housed in USDA approved facilities at the Oklahoma State University-Center for Health Sciences (OSU-CHS), and were acclimated for 1–2 weeks prior to use in experiments. Mice were housed individually in plastic cages (10 × 17 × 28 cm) containing pine chip bedding and paper towel tubes to provide hiding places and environmental enrichment, and had ad libitum access to food and water. The ambient temperature was maintained at 21 °C with a 12:12 light:dark cycle. All experimental manipulations and animal handling procedures were approved by the OSU-CHS Institutional Animal Care and Use Committee.

2.2. Experimental protocol

Mice (n = 6 per group) were administered β-FNA (National Institute on Drug Abuse reagent supply program; 12.5, 25 or 50 mg/kg; i.p.) or saline vehicle (200 μl). Immediately thereafter, mice were administered LPS (*Escherichia coli* O55:B5; Sigma; 0.83 mg/kg; i.p.) or saline vehicle (25 μl). The LPS dose selected is well-established to induce neuroinflammation as well as sickness- and anxiety-like behaviors in mice [14] and the dose range of β-FNA was based on reports in the literature in which β-FNA was administered i.p. in mice [15,16] and our previous findings [13].

2.3. Behavioral measures

Exploratory locomotor activity in the open field test was used as a behavioral marker of acute sickness behavior [14,17]. At 24 h post-treatment, each mouse was placed alone into an open field arena (40 × 40 cm) and behavior was digitally recorded for 10 min. and scored using Ethovision software. The a priori dependent measures were distance (cm) traveled, duration (sec.) in the center zone (the 35 × 35 cm inner region of the arena), duration in the corners (the 10 × 10 cm corner regions of the arena), and number of rearings. Reduction in distance moved and increased time along the walls and in the corners are reliable indicators of sickness behavior [14,17]. Reluctance to move away from the walls is also a measureable form of anxiety-like behavior and rearing activity is a commonly observed exploratory behavior (17).

2.4. Tissue collection

Following open-field testing, mice were euthanized by CO_2 inhalation, after which they were decapitated and trunk blood and brain were collected on water-ice. Plasma was collected after centrifugation and stored at −80 °C. Whole brain tissue was washed one time by adding 0.5 ml ice cold phosphate buffered saline, centrifugation (500 ×*g*, 3 min, 4 °C), and aspiration. Tissue was homogenized in 4.7 ml ice cold triple-detergent lysis buffer [18] using a Sonic Dismembrator Model 100 (Fisher Scientific), then centrifuged (20,000 ×*g*, 30 min, 4 °C). The aqueous phase was collected and stored at −80 °C for subsequent quantification of inflammatory markers. Total protein levels in plasma and brain homogenates, determined using the bicinchoninic acid (BCA) protein assay, were used to normalize data [19].

2.5. Measurement of pro-inflammatory mediators

Standard dual-antibody solid phase immunoassays (ELISA Development Kit; Peprotech) were used to quantify IL-6, CXCL10, and CCL2 in plasma and in whole brain homogenates, according to the manufacturer's instructions and as previously described [10].

2.6. Statistical analysis

Prism™ version 6.07 software (GraphPad Inc., San Diego, CA) and Statistica 12 (Statsoft, Tulsa, OK) were used for statistical analysis and figure presentation. Overall analysis for each dependent measure was performed using two-way analysis of variance (ANOVA) with LPS treatment and β-FNA dose as grouping variables. ANOVAs that revealed a statistically significant interaction were probed further using Tukey's test to examine all possible pair-wise comparisons. In one case, the ANOVA revealed a marginal p–value paired with a moderate effect size. In this case we opted to use a Tukey's test despite the fact that there was no statistically significant interaction. For all other ANOVAs, planned comparisons between the saline and LPS treated groups that did not receive β-FNA were performed using *t*-tests. In addition, we performed post hoc pair-wise comparisons dictated by visual analysis of the data using Fisher's LSD tests. The p-values for these post hoc comparisons were multiplied by the number of comparisons that were made for the respective measures. Linear regression was also performed. Data are reported as mean ± SEM, p-values < 0.05 were considered statistically significant, and effect sizes are reported as η^2_p for ANOVAs and r^2 for regressions.

3. Results

3.1. Effects of β-FNA on LPS-induced cytokine/chemokine expression in plasma and brain

LPS treatment significantly increased plasma levels of IL-6, CCL2 and CXCL10 at 24 h, relative to saline treated controls. In plasma, only CXCL10 expression was significantly affected by β-FNA administration as indicated by inhibition at the 25 mg/kg dose. More specifically, for plasma IL-6 levels, there was a main effect of LPS treatment ($F_{1,33} = 33.92$, $p < 0.001$, $\eta^2_p = 0.51$), but no main effect of β-FNA dose ($F_{3,33} = 1.94$, $p = 0.14$, $\eta^2_p = 0.15$) and no interaction ($F_{3,33} = 0.62$, $p = 0.61$, $\eta^2_p = 0.05$). Planned pair-wise comparisons showed that, absent β-FNA, LPS-treated mice had significantly higher plasma IL-6 levels than did saline-treated control mice (Fig. 1A). Treatment with β-FNA had no influence on LPS-induced changes in plasma IL-6 levels.

There was a main effect of LPS treatment ($F_{1,34} = 140.6$, $p < 0.001$, $\eta^2_p = 0.81$) and a marginal, but not statistically significant main effect of β-FNA administration ($F_{3,34} = 2.87$, $p = 0.051$, $\eta^2_p = 0.20$) on CCL2 in plasma (Fig. 1C). However, there was a significant LPS × β-FNA interaction ($F_{3,34} = 3.63$, $p < 0.03$, $\eta^2_p = 0.24$) on plasma CCL2 levels. At all β-FNA doses, plasma levels of CCL2 were higher in LPS-treated mice than in their saline treated counterparts. In addition, plasma CCL2 levels in LPS-treated mice that received 50 mg/kg β-FNA were higher than in LPS-treated mice that received β-FNA at 12.5 and 25 mg/kg, but did not differ from the LPS alone group.