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(+)-Naltrexone is neuroprotective and promotes alternative activation in the mouse hippocampus after cardiac arrest/ cardiopulmonary resuscitation

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Abstract

Despite dramatic improvement in cardiopulmonary resuscitation (CPR) and other techniques for cardiac arrest (CA), the majority of survivors continue to show signs of decreased memory or executive cognitive function. Such memory impairment may be due to hippocampal CA1 neuronal death, which is delayed by several days after CA/CPR. Classical microgliosis in the CA1 region may contribute to neuronal death, yet the role of a key activation receptor Toll Like Receptor 4 (TLR4) has not been previously investigated for such neuronal death after CA/CPR. We show that (+)-naltrexone was neuroprotective after CA/CPR. TLR4 blockade was associated with decreased expression of markers for microglial/macrophage activation and T cell and B cell infiltration, as well as decreased pro-inflammatory cytokine levels. Notably, IL-10 expression was elevated in response to CA/CPR, but was not attenuated by (+)-naltrexone, suggesting that the local monocyte/microglial phenotype had shifted towards alternative activation. This was confirmed by elevated expression of Arginase-1, and decreased expression of NF κ B p65 subunit. Thus, (+)-naltrexone and other TLR4 antagonists may represent a novel therapeutic strategy to alleviate the substantial burden of memory or executive cognitive function impairment after CA/CPR.

Keywords

TLR4; ischemia; neurotoxicity; M2 microglia; M2 macrophages; HIF1 α ; HIF2 α

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1. Introduction

Cardiac arrest (CA) is a leading cause of mortality in developed nations (Go et al., 2014). Despite dramatic improvement in cardiopulmonary resuscitation (CPR) techniques and the widespread adoption of hypothermia as a treatment option, the majority of survivors continue to show signs of decreased memory or executive cognitive function, which is a cause of significant and sustained disability (Allen and Buckberg, 2012; Cronberg et al., 2009; Mateen et al., 2011). Such memory impairment is believed to be caused by death of hippocampal CA1 neurons, which are especially sensitive to transient global ischemia (Allen and Buckberg, 2012; Garcia, 1988; Garcia and Anderson, 1989). Since neuronal death is delayed by several days after CA/CPR (Bottiger et al., 1998), there is a unique window of opportunity for therapeutic intervention. Despite the fact that trained medical personnel are present during this window, there are no routine interventions to prevent delayed neuronal death after CA/CPR.

Microglia are the tissue specific macrophages of the central nervous system that rapidly respond to insult, sometimes creating a neurotoxic environment (Kettenmann et al., 2011). Microgliosis may occur in response to a broad array of challenges, including ischemia (Kettenmann et al., 2011), and markers of microgliosis are increased in the CA1 region of the hippocampus after CA/CPR (Norman et al., 2011; Wang et al., 2013a). Attenuation of microgliosis and the neurotoxic products of microglia is generally associated with neuroprotection after global ischemia (Neigh et al., 2009; Takeuchi et al., 2008; Tang et al., 2010; Wang et al., 2013a; Webster et al., 2013; Zhang et al., 2012). Moreover, induction of interleukin 10 (IL-10) producing microglia (alternatively activated microglia) (Locati et al., 2013) is also neuroprotective for CA1 hippocampal neurons after CA/CPR (Wang et al., 2013a). Therefore, classical microgliosis may contribute to neuronal death after CA/CPR. One classical activation pathway is the activation of Toll-Like Receptor 4 (TLR4), which is a key receptor by which microglia and macrophages sense danger in their local environment. Upon cell stress or sterile injury, such as that occurring under ischemic conditions, cells release Danger Associated Molecular Patterns (DAMPs), such as extracellular matrix proteins and heat shock proteins that are agonists at TLR4 (Kawai and Akira, 2010). TLR4 signaling is considered to induce classical activation in peripheral macrophages (Locati et al., 2013), and results in the production of a wide range of neurotoxic mediators by microglia (e.g. tumor necrosis factor (TNF) and IL-1 β). We have previously shown that (+)-naltrexone (which does not bind the stereoselective mu opioid receptor), blocks TLR4 signaling and attenuates expression of microglial activation markers and pro-inflammatory cytokine release (Hutchinson et al., 2008). However the neuroprotective capacity of (+)-naltrexone in hippocampal neuronal death after CA/CPR has not been previously addressed. Hence, we aimed to test whether TLR4 blockade by (+)-naltrexone would attenuate CA/CPR-induced neuronal death and microgliosis in the hippocampus.

2. Materials and Methods

2.1 Experimental animals

All experimental protocols were approved by the University of Colorado Denver Institutional Animal Care and Use Committee and conformed to the National Institutes of

Health guidelines for the care and use of animals in research. Adult male C57Bl/6 mice (8–12 week old; Charles River Laboratory,) were used for this study. Mice were housed in temperature- (18–21°C) and light-controlled (12 h light/dark cycle; lights on at 07:00 h) rooms with standard rodent food and water available ad libitum.

2.2 Cardiac arrest, cardiopulmonary resuscitation model, and (+)-naltrexone administration

Male mice were subjected to CA/CPR as previously described (Deng et al., 2014b; Kofler et al., 2004). Briefly, anesthesia was induced with 3% isoflurane and maintained with 1% to 1.5% isoflurane in O₂ enriched air via face mask. Temperature probes were inserted into the left temporalis muscle and rectum to monitor head and body temperature simultaneously. For drug administration, a PE-10 catheter was inserted into the right internal jugular vein and flushed with heparinized 0.9% saline. A second PE-10 catheter was introduced into the right femoral artery and connected to a pressure transducer to continuously monitor mean arterial blood pressure (Gould Instruments, Valley View, OH). Animals were then endotracheally intubated, connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany) and set to a respiratory rate of 160/min. Cardiac arrest was induced by injection of 70 mL cold (4°C) 0.5 M KCl via the jugular catheter, and confirmed by appearance of asystole on the EKG monitor and no spontaneous breathing. During CA, body temperature was cooled to 28°C and the head temperature increased to 38.8°C.

CPR was begun 8 minutes after induction of CA by injection of 0.5 to 1 mL prewarmed epinephrine solution (16 mg/mL, 0.9% saline), chest compressions at a rate of 300/min, and ventilation with 100% oxygen at a respiratory rate of 190/min and a 25% increased tidal volume. Cardiac massage was stopped as soon as spontaneous circulation was restored. Return of spontaneous circulation was assessed by reappearance of electrical activity on the ECG monitor, rapid decreasing of head temperature, and observation of the chest for visible cardiac contractions to ascertain that electrical activity of the heart was accompanied by appropriate mechanical activity. CPR was abandoned if spontaneous circulation was not restored within 2.5 minutes. Additional animals underwent sham surgery. These animals were instrumented and monitored as above, but no potassium chloride or epinephrine was injected.

(+)-Naltrexone (gifted by Dr. Kenner Rice, NIDA/NIAAA) was administered via intraperitoneal injection (0, 3 or 6 mg/kg, 0.9% saline) twice daily for 2 days, beginning 30 minutes after CA. Unlike its stereoisomer, (+)-naltrexone does not bind to opioid receptors, but blocks TLR4 signaling, and readily crosses the blood-brain barrier (Hutchinson et al., 2008).

2.3 Histology

Three days after CA/CPR, mice were deeply anesthetized with Isoflurane (5%), transcardially perfused with 0.9% saline followed by 4% paraformaldehyde and brains collected. Brains were embedded in paraffin and 6 µm coronal sections were cut through the dorsal hippocampus. Sections were stained with hematoxylin and eosin (H&E) for analyses of neuronal injury. The hippocampal CA1 region was analyzed at three anatomic levels, 100

µm apart starting at −1.5 mm from bregma. Nonviable neurons were identified by dark pyknotic nucleus and pink eosinophilic cytoplasm. Percentage of viable and nonviable neurons were counted for the entire CA1 region of each slice and averaged for the three sections/animal, as previously described.

2.4 Western blotting

Three days after CA/CPR, mice were deeply anesthetized with Isoflurane (5%), transcardially perfused with 0.9% saline and the hippocampus dissected. Western blot analyses were performed on hippocampal tissue that was sonicated, on ice, in 50 mM Tris buffer containing 100 mM 6-amino-n-caproic acid, 1 mM EDTA, 5 mM benzamidine, 0.2 mM phenylmethyl sulfonyl fluoride (in 100% ethanol), and protease inhibitors. After extraction, proteins were subjected to NuPAGE Bis-Tris (4–12%) gel electrophoresis under reducing conditions and then transferred to nitrocellulose membranes electrophoretically using an iblot (Invitrogen, Carlsbad, CA). Nonspecific binding sites on the membrane were blocked with Odyssey Blocking Buffer (50%; LI-COR Biosciences, Lincoln, NE) in TBS containing 0.1% Tween-20, 0.05% Tris-Chloride, and 0.03% 5 M NaCl (TBS-T) for 1 h at 22–24°C. Membranes were subsequently incubated with primary antibodies in Odyssey Blocking buffer containing 0.1% Tween-20 overnight at 4°C. The membranes were then washed with PBS containing 0.1% Tween-20, and probed with appropriate IRDye secondary antibodies (LI-COR Biosciences) in Odyssey Blocking buffer containing 0.1% Tween-20 for 1 h at 22–24°C, protected from light. Following washing with PBS containing 0.1% Tween-20, membranes were scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences). Where necessary, membranes were stripped with a NewBlot Stripping Buffer according to manufacturer instructions (LI-COR Biosciences), and re-probed with an antibody against loading control protein. Primary antibodies and dilution ratios used were: mouse CD11b 1:100 (BD Biosciences, San Jose, CA), mouse T Cell Receptor (TCR) 1:1,000 (BD Biosciences), rabbit CD20 1:2,000 (Santa Cruz Biotechnology, Dallas, TX), mouse Arginase-1 1:1,000 (BD Biosciences), rabbit p65 1:500 (Millipore, Billerica, MA), rabbit Integrin alpha 4 1:1,000 (Abcam, Cambridge, MA), rabbit vascular cell adhesion molecule-1 (VCAM-1) 1:400 (Santa Cruz Biotechnology), rabbit HIF1α 1:1000 (Millipore), rabbit HIF2α 1:1000 (Abcam). Mouse β actin 1:100,000 (Sigma, St. Louis, MO) was used against loading control protein. Secondary antibodies used were: Goat anti-mouse IRDye 680RD 1:15,000, (LI-COR Biosciences), Goat anti-rabbit IRDye 800CW 1:15,000, (LI-COR Biosciences). Bands were quantified using Image Studio (LI-COR Biosciences).

2.5 Multiplex cytokine analysis

Multiplex protein quantitation (MSD MULTI-SPOT Assay System, Rockville, MD) was utilized to quantitate 12 mouse analytes from single samples. Specifically the cytokines: Interferon-γ (IFN-γ; detection range: 0.116–764 pg/ml); IL-10 (detection range: 1.03–2430 pg/ml); IL-12p70 (detection range: 12.5–22400 pg/ml); IL-1β (detection range: 0.26–1440 pg/ml); IL-2 (detection range: 0.377–2070 pg/ml); IL-4 (detection range: 0.223–1220 pg/ml); IL-5 (detection range: 0.545–812 pg/ml); IL-6 (detection range: 2.12–3930 pg/ml); CXCL1 (detection range: 0.226–1630 pg/ml); tumor necrosis factor (TNF; detection range: 0.356–481 pg/ml); were quantified from hippocampal sonicates. The protein concentration

from each sample was determined using a Bradford protein assay as described previously (Bradford, 1976), and used to normalize the Multiplex results.

2.6 Statistics

For Western blots, differences between treatment groups were determined using unpaired one-way ANOVAs followed by Tukey's post hoc tests. For multiplex cytokine analyses, differences between treatment groups were determined using two-way ANOVAs followed by Holm Sidak post hoc tests.

3. Results

3.1 (+)-Naltrexone is neuroprotective for hippocampal CA1 neurons after CA/CPR

Male C57Bl/6 mice were subjected to 8 minute CA/CPR, resulting in neuronal injury in the CA1 region of hippocampus that was analyzed 3 days after resuscitation. Immediate asystole was observed in all mice following injection of KCl and were successfully resuscitated within the CPR time window of 2 minutes. We tested the ability of two (+)-naltrexone doses to protect neurons against CA/CPR-induced damage. Mice treated with 3 mg/kg intraperitoneal (+)-naltrexone 30 minutes after CA/CPR (with booster doses administered 6 hours, 24 and 48 hours later) experienced significant protection against ischemic cell death, exhibiting $22.6 \pm 6.3\%$ cell death, compared to $43.2 \pm 6.9\%$ ($P < 0.05$) in vehicle treated mice (Figure 1A-). To assess dose-dependence, mice were administered 6 mg/kg (+)-naltrexone using the same protocol. Mice treated with 6 mg/kg experienced further protection, exhibiting $15.1 \pm 3.9\%$ ($P < 0.05$ compared to vehicle). Note, the 6 mg/kg dose appears to provide greater protection compared to the 3 mg/kg dose, however analysis with 1-Way ANOVA shows no significant difference between the two doses of (+)-naltrexone tested.

3.2 (+)-Naltrexone attenuates pro-inflammatory processes without suppressing IL-10 in the hippocampus after CA/CPR

TLR4 blockade by (+)-naltrexone has previously been shown to attenuate expression of microglial activation markers after CNS injury (Hutchinson et al., 2008). Therefore, CD11b expression was examined and shown to be elevated after CA/CPR ($P < 0.001$), which was significantly attenuated by (+)-naltrexone treatment ($P < 0.001$; Fig. 2A).

Pro-inflammatory cytokines derived from microglia/monocytes IL-1 β ($P < 0.001$), IL-6 ($P < 0.05$), TNF ($P < 0.05$) and CXCL1 ($P < 0.05$), but not IL-12p70, were significantly elevated in response to CA/CPR (Fig. 2B–F). IL-1 β ($P < 0.05$), IL-6 ($P < 0.05$), TNF ($P < 0.05$) and CXCL1 ($P < 0.05$), but not IL-12p70 were attenuated by (+)-naltrexone treatment (Fig. 2B–F). The anti-inflammatory cytokine IL-10 was elevated in response to CA/CPR ($P < 0.05$), but was not attenuated by (+)-naltrexone treatment (Fig. 2G).

3.3 (+)-Naltrexone promotes alternative activation in the hippocampus after CA/CPR

Since IL-10 levels remained unchanged by (+)-naltrexone treatment, despite robust attenuation of CD11b expression (Fig. 2), we investigated alternative activation marker expression. Arginase-1 was not significantly altered by CA/CPR, but was elevated after (+)-naltrexone treatment ($P < 0.01$; Fig. 3A). Also suggestive of decreased pro-inflammatory

activity, the p65 subunit of the transcription factor NF κ B, elevated by CA/CPR ($P < 0.01$), was attenuated by (+)-naltrexone ($P < 0.01$; Fig. 3B). Since Arginase-1 expression is suppressed by HIF-1 α , but increased by HIF-2 α (Chavez and LaManna, 2002; Takeda et al., 2010), we quantified the expression levels of these proteins. HIF-1 α was significantly elevated in response to CA/CPR ($P < 0.05$), and attenuated by (+)-naltrexone treatment (Fig. 3C; $P < 0.05$). In contrast, HIF-2 α was unchanged following CA/CPR, but elevated by (+)-naltrexone treatment (Fig. 3D; $P < 0.01$).

3.4 (+)-Naltrexone attenuates peripheral immune cell trafficking in the hippocampus after CA/CPR

As we have recently shown that lymphocytes are present in the parenchyma after CA/CPR (Deng et al., 2014a), we quantified the expression of several lymphocyte markers herein. The marker for B cell infiltration (CD20), but not T cell infiltration (TCR) was significantly elevated in response to CA/CPR ($P < 0.05$), and the expression of both markers was attenuated by (+)-naltrexone treatment ($P < 0.05$; Fig. 4A, B). Lymphocyte trafficking to the hippocampus was not accompanied by altered expression in the adhesion molecule VCAM-1, or its ligand VLA-4 (data not shown), suggesting entry due to blood-brain barrier (BBB) disruption.

Pro-inflammatory cytokines derived from T cells IFN γ ($P < 0.05$) and IL-2 ($P < 0.05$) were significantly elevated in response to CA/CPR and attenuated by (+)-naltrexone treatment ($P < 0.05$; Fig. 4C,D). The anti-inflammatory cytokines IL-4, IL-5 were not significantly modulated under any treatment conditions (Fig. 4E,F).

4. Discussion

In this study, we show for the first time that TLR4 blockade by (+)-naltrexone is neuroprotective for hippocampal CA1 neurons after CA/CPR. The microglial activation marker CD11b, as well as several pro-inflammatory cytokines were elevated in the hippocampus after CA/CPR, and were decreased by TLR4 blockade. Notably, the only anti-inflammatory cytokine to be modified under any treatment conditions in the hippocampus was IL-10, whose expression level was furthermore unaffected by (+)-naltrexone treatment. This suggests that (+)-naltrexone promoted expression of alternatively activated microglia, which was confirmed by an increase in Arginase-1 expression. A marker for B cell infiltration was elevated in the hippocampus after CA/CPR, and together with a marker for T cell infiltration, were decreased by TLR4 blockade. In line with previous findings (Kaur and Ling, 2008), it is likely that lymphocytes infiltrated the immune system due to disruption of the BBB, rather than by transendothelial migration, since expression levels of neither the lymphocyte adhesion molecule VCAM-1 nor its ligand were altered under any treatment conditions. Though not directly quantified, neutrophils may also migrate to the hippocampus, since its chemotactic factor CXCL1 was also elevated at the site by CA/CPR. The absence of lymphocytes among the mice treated with (+)-naltrexone is supported by a decrease in the corresponding pro-inflammatory cytokine profiles (IFN γ , IL-2).

TLR4 is predominantly expressed by innate immune cells, namely monocytes/macrophages and dendritic cells in the periphery and microglia in the central nervous system. DAMPs

released in the hippocampus under global ischemic conditions, such as heat shock proteins (Truettner et al., 2009), are known endogenous agonists of TLR4. Accordingly, increased expression of microglial activation markers has been observed in the hippocampus after CA/CPR (Norman et al., 2011; Wang et al., 2013a). The consequence of TLR4 signaling is a cellular phenotype consistent with classical activation (Locati et al., 2013), and activation of the transcription factor NF κ B and mitogen activated protein kinases that result in transcription of pro-inflammatory and neurotoxic mediators (Kawai and Akira, 2010). The data presented herein support that TLR4 signaling was induced by CA/CPR, since blockade by (+)-naltrexone was sufficient to attenuate the expression of NF κ B and a wide array of innate immune cytokines.

There are several ways in which blockade of TLR4 signaling may be neuroprotective for hippocampal CA1 neurons. The first is the reduction of pro-inflammatory cytokines, as those studied here are neurotoxic under certain circumstances: TNF can induce neuronal death via the Fas pathway (Gaur and Aggarwal, 2003); and, IL-1 β can indirectly induce glutamate excitotoxicity by downregulating the glutamate transporters on astrocytes (Yan et al., 2014). Though not measured here, nitric oxide is also produced as a consequence of TLR4 signaling (Wang et al., 2013b) and excess levels contribute to nitrosative and oxidative stress (Calabrese et al., 2007). Hence, attenuation of such signaling may have clear neuroprotective effects. It appears likely that lymphocytes are found in the hippocampus after CA/CPR due to disruption of the BBB (Kaur and Ling, 2008). These cells respond to cytokines released by innate immune cells by releasing their own repertoire of pro-inflammatory cytokines, and recognize antigens presented by microglia/macrophages, which is driven in part by TLR4 (Gertig and Hanisch, 2014; Grace et al., 2011; Wilson et al., 2010). Hence, blockade of TLR4 signaling would attenuate lymphocyte activity as a secondary consequence. Peripheral immune cells likely contribute to neurotoxicity, since restoration of BBB integrity also mediates neuroprotection (Lee et al., 2014).

Despite decreasing the expression of CD11b, TLR4 blockade also induced expression Arginase-1 and retained IL-10 levels observed after saline treatment. These data suggest that the population of activated microglia/macrophages had decreased, but those present exhibited an alternative activation phenotype. The mechanisms underlying this transition are not entirely clear, since such polarization typically requires IL-4 signaling (Locati et al., 2013), which was unchanged under any treatment conditions. One possibility is that alternative activation occurred when opposition by pro-inflammatory factors was abrogated by TLR4 blockade. For example, HIF-2 α is responsible for Arginase-1 expression, but its expression is opposed under ischemic conditions by transcription of HIF-1 α and IFN γ (Chavez and LaManna, 2002; Takeda et al., 2010), which was elevated by CA/CPR herein. Thus, if TLR4 blockade suppresses IFN γ production, then Arginase-1 may be produced. Alternatively activated microglia/macrophages may induce neuroprotection via anti-inflammatory cytokine production, such as IL-10 (Kwilasz et al., 2014). Furthermore, the presence of alternatively activated microglia/macrophages may be essential for neuroprotection, as non-selective depletion of microglia by liposome-encapsulated clodronate was not sufficient to prevent neuronal death in the hippocampus after CA/CPR (Drabek et al., 2012).

In this study, we describe a novel therapeutic strategy to attenuate hippocampal CA1 neuronal death after CA/CPR. TLR4 blockade by (+)-naltrexone attenuated such neurotoxicity, possibly by reducing pro-inflammatory processes and inducing alternative activation of microglia/macrophages. The treatment strategy modeled here is clinically relevant, since (+)-naltrexone was systemically administered within the therapeutic window when medical personnel would be present, and poses no impediment to widespread adoption after CA/CPR. Thus, (+)-naltrexone and other TLR4 antagonists may be useful agents to alleviate the substantial burden of memory or executive cognitive function impairment after CA/CPR.

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Research Highlights

- TLR4 blockade by (+)-naltrexone is neuroprotective after CA/CPR
- (+)-Naltrexone decreased expression of CD11b and pro-inflammatory cytokines
- (+)-Naltrexone elevated Arginase-1 expression, an alternative activation marker
- (+)-Naltrexone may clinically alleviate memory impairment after CA/CPR

Figure 1. (+)-Naltrexone reduces neuronal injury following CA/CPR

Representative photomicrographs of CA1 neurons from vehicle (A), 3 mg/kg (B) and 6 mg/kg (C) (+)-Naltrexone treated mice 3 days after CA/CPR. Damaged neurons identified by presence of pink eosinophilic cytoplasm and dark pyknotic nucleus. D) Quantification of ischemic CA1 neurons. $n = 6$ per group. Data are presented as mean $\pm$ SEM. * $P < 0.05$ compared to vehicle.

Figure 2.

(+)-Naltrexone attenuates microglial activation marker expression and pro-inflammatory cytokine levels, and increases anti-inflammatory cytokine levels after CA/CPR in the hippocampus. (A) The marker for microglial/monocyte activation (CD11b) was significantly elevated in response to CA/CPR, and attenuated by (+)-naltrexone treatment. (B–F) Pro-inflammatory cytokines derived from microglia/monocytes: IL-1β, IL-6, TNF and CXCL1, but not IL-12p70, were significantly elevated in response to CA/CPR and attenuated by (+)-naltrexone treatment. (G) IL-10 was elevated in response to CA/CPR, but was not attenuated by (+)-naltrexone treatment. $n = 6-7$ per group. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$: relative to sham; * $P < 0.05$; *** $P < 0.001$: relative to saline.

Figure 3.

(+)-Naltrexone treatment promotes alternative activation after CA/CPR in the hippocampus. (A) Arginase-1 expression was increased, while (B) expression of the NF κ B p65 subunit was decreased by (+)-naltrexone treatment. (C) HIF-1 α expression was increased by CA/CPR and decreased by (+)-naltrexone treatment, while (D) HIF-2 α expression was increased by (+)-naltrexone treatment. $n = 6-7$ per group. ## $P < 0.01$: relative to sham. * $P < 0.05$; ** $P < 0.01$: relative to saline.

Figure 4.

(+)-Naltrexone attenuates lymphocyte activation marker expression and cytokine levels after CA/CPR in the hippocampus. Markers for (A, B) B cell infiltration (CD20), but not T cell infiltration (TCR) were significantly elevated in response to CA/CPR and were attenuated by (+)-naltrexone treatment, including TCR. Pro-inflammatory cytokines derived from T cells (C, D) IFN γ and IL-2 were significantly elevated in response to CA/CPR and attenuated by (+)-naltrexone treatment. The anti-inflammatory cytokines (E, F) IL-4, IL-5 were not significantly modulated under any treatment conditions. $n = 6-7$ per group. # $P < 0.05$: relative to sham; * $P < 0.05$; ** $P < 0.01$: relative to saline.