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GABAergic modulation with classical benzodiazepines prevent stress-induced neuro-immune dysregulation and behavioral alterations

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ABSTRACT

Objective: Psychosocial stress is associated with altered immunity, anxiety, and depression. Repeated social defeat (RSD), a model of social stress, triggers egress of inflammatory myeloid progenitor cells (MPCs; $CD11b^{+}/Ly6C^{hi}$) that traffic to the brain, promoting anxiety-like behavior. In parallel, RSD enhances neuroinflammatory signaling and long-lasting social avoidant behavior. Lorazepam and clonazepam are routinely prescribed anxiolytics that act by enhancing GABAergic activity in the brain. Besides binding to the central benzodiazepine binding site (CBBS) in the central nervous system (CNS), lorazepam binds to the translocator protein (TSPO) with high affinity causing immunomodulation. Clonazepam targets the CBBS and has low affinity for the TSPO. Here the aims were to determine if lorazepam and clonazepam would: (1) prevent stress-induced peripheral and central inflammatory responses, and (2) block anxiety and social avoidance behavior in mice subjected to RSD.

Methods: C57/BL6 mice were divided into experimental groups, and treated with either lorazepam (0.10 mg/kg), clonazepam (0.25 mg/kg) or vehicle (0.9% NaCl). Behavioral data and tissues were collected the morning after the last cycle of RSD.

Results: Lorazepam and clonazepam were effective in attenuating mRNA expression of CRH in the hypothalamus and corticosterone in plasma in mice subjected to RSD. Both drugs blocked stress-induced levels of IL-6 in plasma. Lorazepam and clonazepam had different effects on stress-induced enhancement of myelopoiesis and inhibited trafficking of monocytes and granulocytes in circulation. Furthermore, lorazepam, but not clonazepam, inhibited splenomegaly and the production of pro-inflammatory cytokines in the spleen following RSD. Additionally, lorazepam and clonazepam, blocked stress-induced accumulation of macrophages ($CD11b^{+}/CD45^{high}$) in the CNS. In a similar manner, both lorazepam and clonazepam prevented neuroinflammatory signaling and reversed anxiety-like and depressive-like behavior in mice exposed to RSD.

Conclusion: These data support the notion that lorazepam and clonazepam, aside from exerting anxiolytic and antidepressant effects, may have therapeutic potential as neuroimmunomodulators during psychosocial stress. The reversal of RSD-induced behavioral outcomes may be due to the enhancement of GABAergic neurotransmission, or some other off-target effect. The peripheral actions of lorazepam, but not clonazepam, seem to be mediated by TSPO activation.

1. Introduction

Psychosocial stress promotes brain-to-immune and immune-to-brain communication that can impact neurobiology and behavior. For example, exposure to stress is capable of causing peripheral immune dysregulation and also enhanced neuroinflammatory signaling by repeated activation of neuroendocrine and autonomic pathways. Activation of these pathways may contribute to the

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development of diseases and mental health disturbances. Two of the most prevalent behavioral outcomes induced by psychosocial stressors are anxiety and depression. Both are extremely debilitating and have a great impact on the quality of life. Depression has been reported in patients suffering from anxiety and anxiety has been reported in depressed patients. Both disorders share common symptoms and are associated with increased release of peripheral cytokines (Maes et al., 2011; Hodes et al., 2014).

Repeated social defeat (RSD), a model of psychosocial stress in mice, provides a valuable and predictable probe to study the mechanisms leading to stress-related alterations in inflammation in both the periphery and central nervous system (CNS), and associated anxiety (Kinsey et al., 2007; Wohleb et al., 2011) and depressive-like behaviors (Wohleb et al., 2013). RSD evokes a "fight or flight" response that causes neuronal and microglia activation within brain regions associated with fear, anxiety and threat appraisal, including the pre-frontal cortex, hypothalamus, amygdala, and the CA3 and dentate gyrus of the hippocampus (Wohleb et al., 2015). RSD activates the hypothalamic–pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS), highlighted by increases in systemic glucocorticoids (GC)s that trigger release of catecholamines and pro-inflammatory cytokines (Avitsur et al., 2001; Hanke et al., 2012). RSD promotes increased levels of corticosterone in serum and IL-6 in plasma (Hanke et al., 2012). In addition, published studies indicate, RSD enhances myelopoiesis and promotes the development, priming, and egress of glucocorticoid (GC) resistant $CD11b^{+}/Ly6C^{high}$ myeloid progenitor cell (MPC) population from the bone marrow (BM) to spleen, lung and circulation (Engler et al., 2004; Curry et al., 2010; Powell et al., 2013). Moreover, RSD-induced neuronal and microglia activation triggers the production of pro-inflammatory molecules that promote the development of a reactive endothelium (Wohleb et al., 2015). Vascular brain endothelial cells increase cell adhesion molecule expression, which facilitates the adherence and extravasation of peripherally derived monocytes that differentiate into perivascular and parenchymal macrophages (Wohleb et al., 2015). The increased accumulation of macrophages in the CNS elicited by RSD, enhanced neuroinflammatory signaling. Neuroinflammatory mediators such as IL1-β, TNF-α, and IL-6, are related in the neurobiological changes that reinforce fear/anxiety and threat circuitry (Wohleb et al., 2015), promoting the development and maintenance of anxiety-like behavior (Reader et al., 2015).

Interestingly, recent findings from our laboratory showed that RSD-induced anxiety-like behavior persisted for at least 8 days after social defeat but was resolved by 24 days, when markers of immune alterations associated with RSD, such as splenomegaly, plasma IL-6, and the number of circulating $CD11b^{+}$ cells returned to control levels.

It should be noted, nevertheless, that social avoidance behavior developed after 1 cycle of social defeat (Wohleb et al., 2014), and long-lasting social avoidance to an aggressor was present 24 days after stress cessation. Previous findings from our laboratory (Wohleb et al., 2014; Ramirez et al., 2015) suggest social avoidance is associated with sensitized microglia. For example, at 24 days after stress termination, microglia from RSD mice have a primed phenotype, with an increased production of pro-inflammatory cytokines in response to mitogen stimulation (Ramirez et al., 2015). These findings suggest that social avoidance behavior is related to neuronal activation and microglia stimulation.

In order to relieve anxiety and depression accompanying stress, physicians resort to anxiolytic drugs, such as benzodiazepines (BDZs). The action of BDZs depends on the activation of binding sites, such as the central benzodiazepine binding site (CBBS) and the translocator protein (TSPO). The CBBS is present in several regions of the CNS and is a component of the GABA-A receptor (Papadopoulos et al., 2006). Binding to this site, enhances GABAergic neurotransmission (Ferrarese et al., 1993), which plays an inhibitory effect in the CNS by counteracting stress-induced hyperactivation of the HPA axis. BDZs also stimulate neuroendocrine effects that seem to be mediated at the hypothalamic and/or suprahypothalamic level by suppressing the production of corticotropin-releasing hormone (CRH) (Arvat et al., 2002). On the other hand, the TSPO is structurally and functionally different from the GABA-A receptor. TSPO is mostly, but not only, located in peripheral tissues, (e.g., heart, kidney, liver, lung, and adrenal glands (Papadopoulos et al., 2006). Specifically, the TSPO is present in the mitochondria coupled to an anion channel, in platelets, lymphocytes, mononuclear cells, endothelium, vascular smooth muscle, mast cells, and also in microglia and neurons. Several *in vitro* studies have shown that BDZs that bind to the TSPO can affect cellular and immune functions by suppressing cytokine secretion (Taupin et al., 1991; Kim et al., 2006; Joo et al., 2009; Yousefi et al., 2013), modifying cell proliferation, affecting immune cell migration (Ruff et al., 1985) and cellular phagocytic activity (Jin et al., 2013). Immune cells express GABA receptor transcripts and it has been reported GABA treatment decreases the production of pro-inflammatory cytokines in peripheral macrophages (Bhat et al., 2010).

Taken together, these clues prompted us to explore the use of lorazepam and clonazepam in RSD, to evaluate the effect of these two drugs on the neuroimmune system during psychosocial stress. Lorazepam and clonazepam are BDZs that act as positive allosteric modulators of GABA action with similar pharmacological mechanisms and share anxiolytic properties (Verleye et al., 2008; Saari et al., 2011). These two drugs have been used extensively to distinguish between the CBBS and the TSPO (Casellas, 2002). Lorazepam acts directly on the GABAA-R complex, and has high affinity for the TSPO. On the other hand, clonazepam is a selective agonist of the GABAA-R (CBBS) but does not bind with high affinity to the TSPO (Anholt et al., 1986). These two different positive allosteric modulators of the GABAA-R produce enhancement of GABAergic activity (Möhler, 2006; Uusi-Oukari and Korpi, 2010) by locking the GABA receptor complex into a conformational change that is able to bind GABA with high affinity (Nutt and Malizia, 2001; Saari et al., 2011). Since both drugs affect functions of the endocrine system, it would be logical to expect that these two drugs might modulate the neuroendocrine regulation of inflammation during psychosocial stress by decreasing stress-induced neuroinflammatory signaling, thus, modifying stress-associated behavioral changes. Therefore, the aims in the present study were to determine if lorazepam and clonazepam would (1) prevent stress-induced peripheral and central inflammatory responses, and (2) block anxiety and social avoidance behavior in mice subjected to RSD.

2. Materials and methods

2.1. Animals

Male C57BL/6 (6–8 weeks old) and CD-1 (12 months, retired breeders) mice were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts) and allowed to acclimate to their surroundings for 7–10 days prior to initiation of experiments. C57BL/6 mice were housed in cohorts of three and CD-1 mice were singly housed in 11.5 × 7.5 × 6 inch polypropylene cages. Mice were maintained at 21 °C under a 12:12 h light: dark cycle with *ad libitum* access to water and rodent chow in the animal facility at The Ohio State University. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.