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Development of a primary microglia screening assay and its use to characterize inhibition of system x_c^- by erastin and its analogs

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ABSTRACT

The inflammatory response in the central nervous system involves activated microglia. Under normal conditions they remove damaged neurons by phagocytosis. On the other hand, neurodegenerative diseases are thought to involve chronic microglia activation resulting in release of excess glutamate, proinflammatory cytokines and reactive oxygen species, leading to neuronal death. System x_C^- cystine/glutamate antiporter (SXC), a sodium independent heterodimeric transporter found in microglia and astrocytes in the CNS, imports cystine into the cell and exports glutamate. SXC has been shown to be upregulated in neurodegenerative diseases including multiple sclerosis, ALS, neuroAIDS Parkinson's disease and Alzheimer's disease. Consequently, SXC inhibitors could be of use in the treatment of diseases characterized by neuroinflammation and glutamate excitotoxicity. We report on the optimization of a primary microglia-based assay to screen for SXC inhibitors. Rat primary microglia were activated using lipopolysaccharides (LPS) and glutamate release and cystine uptake were monitored by fluorescence and radioactivity respectively. LPS-induced glutamate release increased with increasing cell density, time of incubation and LPS concentration. Conditions to screen for SXC inhibitors were optimized in 96-well format and subsequently used to evaluate SXC inhibitors. Known SXC inhibitors sulfasalazine, S-4CPG and erastin blocked glutamate release and cystine uptake while R-4CPG, the inactive enantiomer of S-4CPG, failed to inhibit glutamate release or cystine transport. In addition, several erastin analogs were evaluated using primary microglia and found to have EC_{50} values in agreement with previous studies using established cell lines.

1. Introduction

Inflammation in the central nervous system (CNS) is thought to play an important role in the pathogenesis of neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, HIV-1 associated neurocognitive disorders, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and stroke. The inflammatory response is mediated by activated microglia, the resident immune cells of the CNS. Microglial cells represent 10–20% of the cellular population in the CNS and similar to their peripheral counterparts, the macrophages, microglia behavior and morphology are highly dependent on their microenvironment [1,2]. Under normal conditions microglial cells are partly responsible for innate immunity in the CNS; microglia respond to neuronal damage and remove damaged cells by phagocytosis. On the other hand, chronic activation of microglia may cause neuronal damage involving excess glutamate release. Excess glutamate release is thought to induce excitotoxicity through increased activation of neuronal glutamate receptors, increased calcium intake and neuronal apoptosis [3,4].

One glutamate transporter that contributes to nonvesicular release

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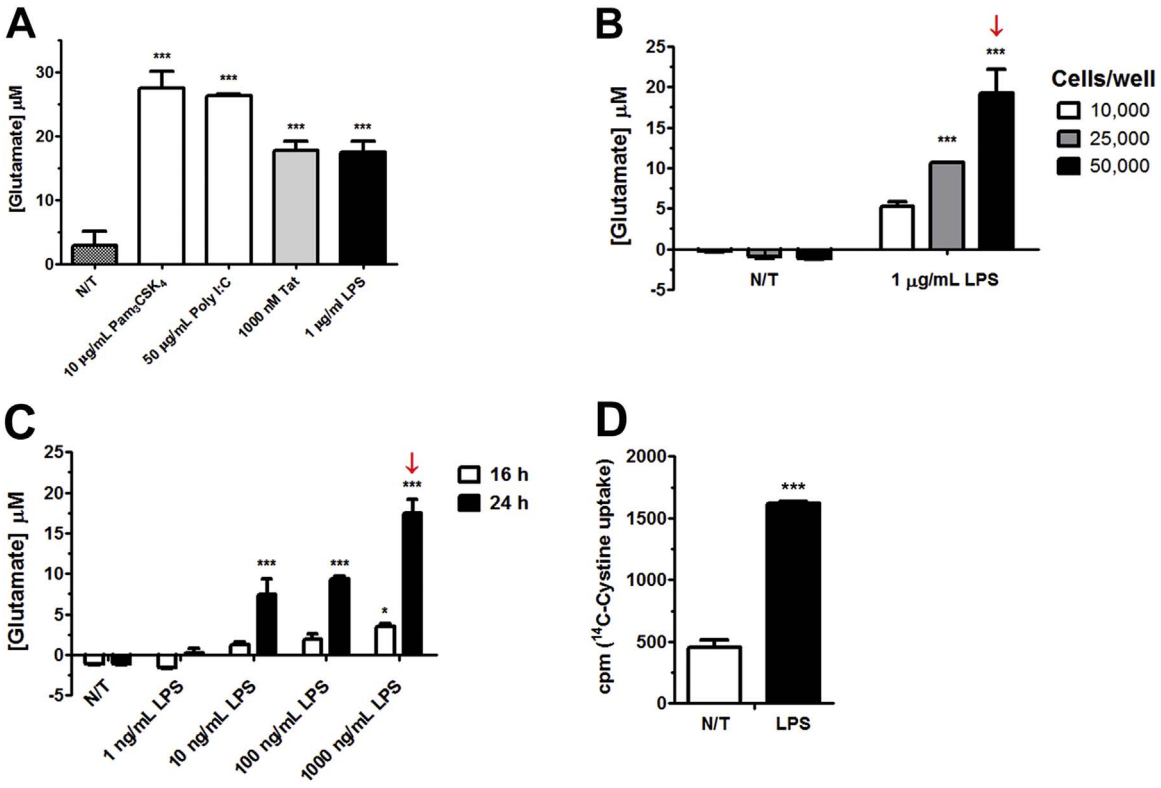

Fig. 1. Characterization of microglia activation assay in 96-well format - (A) Induction of glutamate release from rat primary microglia after 24 h stimulation with Pam_3CSK_4 (10 µg/ml), poly I: C (50 µg/ml), Tat (1 µM) or LPS (1 µg/ml). **(B)** Dependence of glutamate release on cell density after 24 h incubation with LPS (1 µg/ml). **(C)** Dependence of glutamate release on LPS-concentration and time of incubation. Arrows indicate the number of cells (50,000 cells), concentration of LPS (1 µg/ml) and incubation time (24 h) chosen for the 96-well plate microglia activation assays. **(D)** Cystine uptake by primary microglia after 1 µg/ml LPS treatment for 24 h. N/T: no treatment. Specific rate of cystine uptake in microglial cells in the absence of inhibitors from three independent experiments conducted in duplicate was 0.015 ± 0.002 nmol/mg/min. Statistically significant differences (*$p < 0.05$ and ***$p < 0.001$) correspond to a comparison to no treatment (N/T) group.

of glutamate is the cystine/glutamate antiporter SXC [5–7]. This is a Na^+-independent, Cl^--dependent antiporter [5] expressed in a wide variety of cells in the CNS where it plays a key role in glutamatergic neurotransmission as a result of its ability to release glutamate [8–10]. Most recently it has been shown that SXC is a key factor in maintenance of cell homeostasis in oligodendrocytes [11]. SXC is also the transporter largely responsible for glutamate release in activated microglia associated with certain brain pathologies [12–14] and it is a potential drug target for neuroinflammatory diseases associated with excess extracellular glutamate [15]. SXC imports cystine and exports glutamate in a 1:1 ratio [5,16]. However, a recent study shows that in astrocytoma cancer cells (CCF-STTG1) the ratio of cystine uptake to glutamate release could be 10:1 [15]. SXC has been associated with neurological diseases such as AD [17], MS [18,19], ALS [8], neuroAIDS [20] and PD [21,22] and its expression can be enhanced in cell culture by LPS [19] and cytokines like IL-1β [23]. Once cystine is transported inside the cell by SXC, it is reduced to cysteine, which is then utilized for synthesis of the antioxidant tripeptide glutathione (GSH) [24]. GSH is essential for restoring intracellular redox balance when ROS is produced [12]. Inhibition of SXC with small molecules such as (*S*)-4-carboxyphenyl glycine ((*S*)-4-CPG), L-α-aminoadipic acid, sulfasalazine and erastin cause both protective and toxic effects in a variety of cells and *in vivo* systems [16,25–28]. On the positive side, therapeutic inhibition of SXC in cells such as microglia and macrophages protect against neuronal cell death due to exposure to high extracellular glutamate levels and excessive stimulation of glutamate receptors [12,21]. SXC activity has also been associated with various aspects of cancer, including growth and metastasis, glutathione dependent drug resistance and excitotoxicity. Inhibition or deficiency of the transporter leads to cystine starvation and cancer cell growth arrest, cell death and/or enhanced sensitivity to chemotherapy [29]. Although promising as a therapeutic target, there has been a concern that cystine starvation and reduced GSH levels could have detrimental effects in non-cancerous cells exposed to oxidative stress [30]. However, SXC knockout (KO) animals develop normally and are fertile [31]. Further, studies with SXC inhibitors and siRNA have shown good tolerability [32,33]. Given this, SXC has become a target of interest so that monitoring glutamate release during microglia activation and evaluating the effect of potential drugs on blocking this release is of major interest in drug discovery.

Even though primary microglia have previously been used to evaluate SXC inhibitors [18,34–36], the assay has not been thoroughly characterized or implemented to enable moderate to high throughput screening for SXC inhibitors. Herein, we report for the first time, on the characterization of a 96-well format primary microglia-based assay to screen for SXC inhibitors. The assay was systematically characterized for activation of primary microglia as measured by LPS-induced glutamate release with respect to different activating agents, cell density and time of incubation. Subsequently, we used the optimized assay to assess a correlation between glutamate release and cystine uptake using erastin analogs.

2. Materials and methods

2.1. Reagents

Lipopolysaccharides (LPS) from *Escherichia coli* strain O111:B4, erastin, polyinosinic: polycytidylic acid (poly I:C), Pam_3CSK_4, apigenin, buthionine sulphoximine (BSO) and cystine were purchased from Sigma-Aldrich. Cell culture reagents and supplies were purchased from Life Technologies and Greiner Bio One respectively. HIV1- Tat_{1-86} protein was obtained from Diatheva.