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# Mitochondrial dysfunction and lipid peroxidation in rat frontal cortex by chronic NMDA administration can be partially prevented by lithium treatment

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ABSTRACT

Chronic N-methyl-D-aspartate (NMDA) administration to rats may be a model to investigate excitotoxicity mediated by glutamatergic hyperactivity, and lithium has been reported to be neuroprotective. We hypothesized that glutamatergic hyperactivity in chronic NMDA injected rats would cause mitochondrial dysfunction and lipid peroxidation in the brain, and that chronic lithium treatment would ameliorate some of these NMDA-induced alterations. Rats treated with lithium for 6 weeks were injected i.p. 25 mg/kg NMDA on a daily basis for the last 21 days of lithium treatment. Brain was removed and frontal cortex was analyzed. Chronic NMDA decreased brain levels of mitochondrial complex I and III, and increased levels of the lipid oxidation products, 8-isoprostane and 4-hydroxynonenal, compared with non-NMDA injected rats. Lithium treatment prevented the NMDA-induced increments in 8-isoprostane and 4-hydroxynonenal. Our findings suggest that increased chronic activation of NMDA receptors can induce alterations in electron transport chain complexes I and III and in lipid peroxidation in brain. The NMDA-induced changes may contribute to glutamate-mediated excitotoxicity, which plays a role in brain diseases such as bipolar disorder. Lithium treatment prevented changes in 8-isoprostane and 4-hydroxynonenal, which may contribute to lithium's reported neuroprotective effect and efficacy in bipolar disorder.



## 1. Introduction

Excessive glutamate signaling can induce neuronal dysfunction and cell death by producing excitotoxicity, a process that is characterized by a large influx of calcium, resulting in a cascade of events involving disruption of calcium-dependent cellular pathways, mitochondrial dysfunction, production of oxidative stress and activation of apoptosis (Plitman et al., 2014). Glutamate excitotoxicity is hypothesized to be an important contributor in many neurodegenerative and neuropsychiatric disorders, including Parkinson's disease, Alzheimer's disease, Huntington's disease, schizophrenia, and bipolar disorder (BD) (Mehta et al., 2013; Plitman et al., 2014).

Glutamate signaling occurs through ionotropic or metabotropic glutamate receptors. The N-methyl-D-aspartate (NMDA) receptor is an ionotropic glutamate receptor that causes an influx of calcium upon activation, giving it its convulsant properties in high doses due to a dramatic increase in action potentials which can be seen as spike trains in EEG recordings (Corner et al., 2002; Ormandy et al., 1991). Activation of NMDA receptors can also result in the activation of various signaling pathways that are calcium-dependent, including activation of protein kinase C (Fukunaga et al., 1992) and release of arachidonic acid from membrane phospholipid (Dingledine et al., 1999; Lazarewicz et al., 1990; Lazarewicz et al., 1988; Weichel et al., 1999). Interestingly, chronic treatment of rats with mood stabilizers commonly used in BD, including lithium,

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at therapeutically relevant doses decreased alterations in brain arachidonic acid signaling produced by acute NMDA administration (Rapoport, 2014), suggesting that lithium may be able to ameliorate the effects of excitotoxicity produced by chronic hyperactivation of glutamatergic signaling. In this regard, a model for chronic excitotoxicity has been developed, involving daily i.p. injection of NMDA in rats at daily doses ranging from 25 to 130 mg/kg (Ormandy et al., 1991). The lowest dose produced brief spike trains 5–10 s duration within 10 min, which soon disappeared, suggesting that while the calcium influx produced by the dose is sufficient to produce excitation, it is not sufficient to produce convulsions (Ormandy et al., 1991). Pre-treatment with lithium did not potentiate the severity of EEG changes caused by chronic NMDA. We thought that the low dose NMDA regimen would be of interest in this study.

Excessive calcium influx caused by hyperactivation of NMDA receptors may cause oxidative stress by increasing calcium entry into the mitochondria (Clay et al., 2010; Gao et al., 2007; Giorgi et al., 2011; Hama-Tomioka et al., 2012; Lemasters et al., 2009). In turn, excessive mitochondrial calcium can disturb the electron transfer process, producing reactive oxygen species (ROS) through leakage of electrons from the electron transport chain (ETC), particularly complexes I and III. Furthermore, excess calcium can cause activation of nitric oxide synthase, resulting in increased production of nitric oxide that can form peroxynitrite, a reactive nitrogen species (RNS) (Girouard et al., 2009; Hama-Tomioka et al., 2012). RNS and ROS produced in this process can react with lipids, proteins and DNA to cause various structural and functional modifications (Barzilai and Yamamoto, 2004; Beal, 2002; Clay et al., 2010; Fariss et al., 2005). Lipid peroxidation, which can be produced by ROS, was found to correlate with white matter abnormalities in patients with BD (Versace et al., 2014). Lipid peroxidation may be a particularly relevant target to excitotoxicity produced by hyperactivation of NMDA receptors, since chronic NMDA treatment was found to increase the release of arachidonic acid, which is a favorable substrate for lipid peroxidation (Axelrod, 1990; Bosetti et al., 2002; Chang et al., 1996; Felder et al., 1990; Pellerin and Wolfe, 1991).

Therefore, the aim of this study was to examine whether excessive and chronic activation of NMDA receptors could impair functioning of the mitochondrial ETC and lipid peroxidation in the brain. First, we examined whether alterations in the mitochondrial ETC and products of lipid peroxidation occurred in the frontal cortex of rats that were given chronic (21 days) daily subconvulsive intraperitoneal (i.p.) injection of NMDA. Second, we determined if chronic lithium, at a therapeutically relevant dose that is used to treat BD, could decrease these alterations if they occurred.

## 2. Methods

### 2.1. Animals

Experiments were conducted following the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication No. 86-23). Experiments were approved by the Animal Care and Use Committee of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), under Protocol 09-026. Male Fisher CDF (F-344) rats (2 months old) weighing between 200 and 250 g (Charles River Laboratories; Wilmington, MA, USA) were divided into four experimental groups. They were housed in an animal facility that had regulated temperature, humidity and a 12 h light/12 h dark cycle.

Two groups of rats were given lithium followed by NMDA treatment (Group I and II), and two groups (Group III and IV) received regular food followed by NMDA treatment. More specifically, animals in group I (Li + saline; N = 12) and II (Li + NMDA; N = 12) were fed Purina Rat Chow (Harlan, Telkad, Madison, WI, USA) ad libitum containing 1.70 g lithium chloride per kg in pelleted form for 4 weeks, followed by pellets containing 2.55 g lithium chloride per kg for an additional 2 weeks. This feeding regimen has been shown to produce plasma and brain lithium levels of 0.7 mM, which is therapeutically relevant to the treatment of BD (Bosetti et al., 2002; Chang et al., 1996). Rats in groups III (Controls; N = 12) and IV (NMDA; N = 11) received lithium-free Purina Rat Chow for 6 weeks. After 3 weeks on the diet regimen, rats in groups I and III received daily i.p. injections of 0.3 mL 0.9% NaCl for 21 days, while rats in groups II and IV received daily i.p. injections of NMDA solution (25 mg/kg body weight in 0.9% NaCl; Sigma Aldrich, St. Louis, MO, USA) for 21 days. As it has been demonstrated that NMDA mediates alterations in circadian rhythm (Shibata et al., 1994), we were sure to keep the injections at the same time to minimize the effect of any shifts in the circadian rhythm on our results. We also maintained the 12 h night/dark cycle throughout the treatment. This regimen of NMDA does not produce convulsions, but produces brief spike trains that disappear by 5 min after an injection (Ormandy et al., 1991). Three hours after the last injection, animals were sacrificed by overexposure to carbon dioxide and their brains were rapidly removed. The frontal cortex was isolated and flash frozen with methylbutane and dry ice for storage in −80 °C. The frozen samples were sent immediately by FEDEX in a dry ice container to the laboratory of Dr. Andreazza at the University of Toronto, for chemical analysis.

### 2.2. Mitochondrial electron transport chain complex I, III, and V

Levels of ETC complexes I, III and V were measured using the Rat/Mouse Oxidative Phosphorylation (OXPHOS) Magnetic Bead Panel (RM0XPSMAG-17K; EMD Millipore, St. Charles, Missouri, USA) with the Milliplex method following manufacturer's instructions. Briefly, samples were lysed using the lysis buffer provided in the kit and centrifuged to obtain the proteins. 5 ug of total protein was used. After the addition of the samples to the plate, antibody-containing beads were added to the samples for 2 h at room temperature, followed by the addition of detection antibodies for 1 h at room temperature. Streptavidin-phycoerythrin was added to the plate, and the samples were read on Luminex and analyzed using the xPONENT software. Results are expressed in median fluorescence intensity (MFI).

### 2.3. Lipid hydroperoxides

Lipid hydroperoxides were measured using the Lipid Hydroperoxide Assay Kit (705003; Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. With this kit, hydroperoxides are measured using the reaction between hydroperoxides with ferrous ions to produce ferric ions, which can be detected using a chromogen. Briefly, lipid hydroperoxides in homogenized samples (100 ug total protein per sample) were extracted using chloroform and Extract R saturated methanol provided in the kit. The amount of lipid hydroperoxides was measured by adding chromogen to each sample and reading the absorbance at 500 nm using a micro-plate reader (BioTek® Instruments, Winooski, VT, USA).

### 2.4. 8-isoprostane

8-Isoprostane levels were measured using the 8-isoprostane EIA kit (516351; Cayman) according to the manufacturer's instructions. Using this kit, 8-isoprostane levels are measured by competitive binding between 8-isoprostane in the sample and 8-isoprostane-