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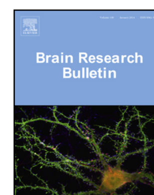

Research report

Corticosterone enhances N-methyl-D-aspartate receptor signaling to promote isolated ventral tegmental area activity in a reconstituted mesolimbic dopamine pathway

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ABSTRACT

Elevations in circulating corticosteroids during periods of stress may influence activity of the mesolimbic dopamine reward pathway by increasing glutamatergic *N*-methyl-D-aspartate (NMDA) receptor expression and/or function in a glucocorticoid receptor-dependent manner. The current study employed organotypic co-cultures of the ventral tegmental area (VTA) and nucleus accumbens (NAcc) to examine the effects of corticosterone exposure on NMDA receptor-mediated neuronal viability. Co-cultures were pre-exposed to vehicle or corticosterone (CORT; 1 μM) for 5 days prior to a 24 h co-exposure to NMDA (200 μM). Co-cultures pre-exposed to a non-toxic concentration of corticosterone and subsequently NMDA showed significant neurotoxicity in the VTA only. This was evidenced by increases in propidium iodide uptake as well as decreases in immunoreactivity of the neuronal nuclear protein (NeuN). Co-exposure to the NMDA receptor antagonist 2-amino-7-phosphonovaleric acid (APV; 50 μM) or the glucocorticoid receptor (GR) antagonist mifepristone (10 μM) attenuated neurotoxicity. In contrast, the combination of corticosterone and NMDA did not produce any significant effects on either measure within the NAcc. Cultures of the VTA and NAcc maintained without synaptic contact showed no response to CORT or NMDA. These results demonstrate the ability to functionally reconstitute key regions of the mesolimbic reward pathway *ex vivo* and to reveal a GR-dependent enhancement of NMDA receptor-dependent signaling in the VTA.

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1. Introduction

Psychological stress often contributes to substance abuse and the development of drug dependence (Jacobsen et al., 2001). The glucocorticoid cortisol is a neuroactive hormone product of hypothalamic–pituitary–adrenal (HPA) axis stimulation that may influence synaptic physiology in the mesocorticolimbic dopamine pathway projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) and other regions. Tidey and Miczek (1996) showed that animals exposed to a tail pinch had increased mesolimbic dopamine levels. Similarly, extracellular levels of dopamine in the NAcc shell were increased in rats immediately following a mild footshock stressor (Kalivas and Duffy, 1995). Removal of endogenous glucocorticoids, *via* adrenalectomy, resulted in decreased extracellular dopamine concentrations in the NAcc shell at baseline and following a stress or cocaine challenge (Barrot et al., 2000). Administration of a glucocorticoid receptor (GR) antagonist resulted in decreased basal dopamine levels in a dose-dependent manner in the same area (Marinelli et al., 1998). Piazza et al. (1993) showed that animals self-administered corti-

Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; APV, DL-2-amino-7-phosphonovaleric acid; Ca^{2+}, calcium; CORT, corticosterone; CRH, corticotrophin releasing hormone; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocynate; GR, glucocorticoid receptor; HPA, hypothalamus–pituitary–adrenal; MEM, minimum essential medium; MR, mineralocorticoid receptor; NA, ccnucleus accumbens; NeuN, neuronal nuclear protein; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PI, propidium iodide; VTA, ventral tegmental area.

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costerone (CORT) at concentrations achieved in serum after stress. Thus, while the importance of CORT elevations in this pathway is not clearly understood, it may well reflect regulatory modulation of reward pathways at rest and/or following exposure to stress-inducing stimuli that is mediated by GRs. However, the underlying signaling mechanisms involved have not been elucidated.

Glucocorticoid modulation of glutamate receptor activity may contribute to GR-mediated effects in specific subregions of the mesolimbic reward pathway. Chronic CORT treatment has been shown to increase mRNA levels of GluN2A and GluN2B subunits of *N*-methyl-D-aspartate (NMDA) receptors in several models systems (Cohen et al., 2011; Costa-Nunes et al., 2014; Prendergast and Mulholland, 2012; Weiland et al., 1997). These ionotropic receptors are highly permeable to Ca^{2+} and promote Ca^{2+}-dependent excitotoxicity in models of ischemia/hypoxia, traumatic brain injury, and ethanol withdrawal (for review, see Lau and Tymianski, 2010). Functionally, these effects of CORT are associated with prolonged NMDA receptor mediated Ca^{2+} signaling (Takahashi et al., 2002; Xiao et al., 2010) and increased sensitivity of NMDA receptor populations to agonists in hippocampal cultures, resulting in pyramidal cell loss in a GR-dependent manner (Mulholland et al., 2004a,b, 2006). Previous studies have indicated that chronic exposure to elevated, yet physiologically-relevant, concentrations of CORT may result in enhanced vulnerability to glutamatergic insults in the hippocampus, implicating these systems in stress-associated cognitive decline (Mulholland et al., 2004a; Takahashi et al., 2002; Wiegert et al., 2005). The current study sought to determine if CORT pre-exposure also enhances NMDA-induced signaling and neurotoxicity in of the mesolimbic reward pathway using an in vitro reconstitution model.

2. Materials and methods

2.1. Organotypic co-culture preparation

In vitro work is well suited to examine CORT-induced differences, as *in vivo* studies with rodents are confounded by diurnal fluctuations in CORT concentrations and *in vitro* models allow for precise control over CORT concentrations. In addition, though the effects of *in vitro* aging on receptor density have not yet been examined in co-cultures of the NAcc and VTA, Martens and Wree (2001) noted that NMDA receptor distribution was comparable in hippocampal slices aged *in vitro* and those taken from aged-matched rats and previous studies have found that a number of synaptic components remain at a steady level following a brief initial depression (Bahr et al., 1995). Additionally, numerous studies have investigated the use of long-term organotypic co-cultures of the VTA/NAcc and reported in tact morphology, re-innervation of dopaminergic tracts into the NAcc, and survival up to 3 months (Jaumotte and Zigmond, 2005; Lyng et al., 2007; Ostergaard et al., 1990). Thus, organotypic cell culture studies allow for long-term and detailed manipulations investigating mechanisms of brain activity while minimizing environmental influences and may provide a better understanding of CORT-NMDA interactions *in vivo* . Eight day old male and female Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) were humanely sacrificed and the brains aseptically removed. Following removal, brains were transferred to ice-cold dissecting media (4 °C), composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 μM streptomycin/penicillin (Invitrogen). Following aseptical removal, brains were sliced mid-sagitally and sectioned coronally at 400 μm thickness using a McIllwain Tissue Chopper (Mickle Laboratory Engineering Co., Ltd., Gomshall, UK). After sectioning, slices were placed into culture medium, containing dissecting medium along with distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% (v/v) Hanks' Balanced Salt Solution (HBSS; Invitrogen) and 25% heat-inactivated horse serum (HIHS; Sigma) and 0.05% streptomycin/penicillin (Invitrogen). Using a dissecting microscope, intact slices containing the NAcc and VTA were identified, yielding approximately 4–6 slices of each region per animal. Two pair of co-cultures (each pair containing one NAcc and one VTA in slight contact) was plated onto Millicell-CM 0.4 μm biopore membrane inserts (Fisher) with 1 mL of pre-incubated culture media added to the bottom of each well of a six well plate. Using the dissecting microscope, slices were oriented such that the NAcc and VTA were in direct contact with each other. Additional cultures were obtained from each region and plated with one slice in each well (i.e., slices not co-cultured) to test the hypothesis that synaptic reconnection was required to produce functional responses to experimental manipulations. Plates were then incubated at 37 °C with a gas composition of 5%CO_2/95% air for 21 days to allow the co-cultures to grow together and attach to inserts. During this time, the culture media was refreshed every 3 days. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

2.2. Characterization of co-culture model

Several measures were taken in order to better characterize the NAcc/VTA co-cultures. Autoradiography was conducted for quantification of dopamine transporter binding sites in control-treated cultures, after Maragos et al. (2002) without modification. Briefly, binding was carried out after 21 days of culturing to match the *in vitro* aging cultures employed in drug exposure studies (below). High pressure liquid chromatography (HPLC) analysis of extracellular dopamine in co-cultures was assessed after Meyer et al. (2013) without modification, with the exception that 1 mL of artificial cerebrospinal fluid was placed on top of each insert for collected 45 min prior stabilization for HPLC analysis. In addition, immunohistochemistry for tyrosine hydroxylase (the rate limiting enzyme in the synthesis of dopamine) and myelin basic protein (to stain myelinated axon fibers) was also conducted on control-treated co-cultures. To assess immunoreactivity, co-cultures were fixed by transferring the insert containing the co-culture to a plate containing 1 mL of 10% formalin solution. One milliliter of formalin was also placed on top of the insert and the plates were allowed to sit for 30 min. The slices were then washed with 1 X phosphate buffered saline (PBS) twice and stored with 1 mL of 1 X PBS overnight at 4 °C. Following overnight storage, the inserts were transferred to a plate containing 1 mL of permeabilization buffer (200 mL 1 X PBS (Invitrogen), 200 μL Triton X-100 (Sigma), 0.010 mg Bovine Serum (Sigma)) in each well and 1 mL of permeabilization buffer was also placed on top of each insert containing the slices for 45 min. Tissue was then washed twice with 1 X PBS. Inserts were then transferred to a plate containing 1 mL of 1 X PBS on the bottom of each well and were treated with 1 mL of permeabilization buffer containing mouse anti-tyrosine hydroxylase (1:200; Sigma) and rabbit anti-myelin basic protein (1:100; Sigma) on top of each well. Plates were then stored at 4 °C for 24 h. The slices were washed gently with 1 X PBS twice and were transferred to a plate containing 1 mL of 1 X PBS on bottom. At this point, slices were treated with 1 mL of permeabilization buffer containing the goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC; 1:100; Sigma) for tyrosine hydroxylase-labeled cultures and the goat anti-rabbit secondary antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC; 1:200; Sigma) for myelin basic protein-labeled cultures on top of the insert and were stored at 4 °C for 24 h. Slices were then washed twice with 1 X PBS and placed into

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