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Original Contribution

Acute restraint stress induces specific changes in nitric oxide production and inflammatory markers in the rat hippocampus and striatum

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ABSTRACT

Chronic mild stress has been shown to cause hippocampal neuronal nitric oxide synthase (NOS) overexpression and the resultant nitric oxide (NO) production has been implicated in the etiology of depression. However, the extent of nitrosative changes including NOS enzymatic activity and the overall output of NO production in regions of the brain like the hippocampus and striatum following acute stress has not been characterized. In this study, outbred male Wistar rats aged 6–7 weeks were randomly allocated into 0 (control), 60, 120, or 240 min stress groups and neural regions were cryodissected for measurement of constitutive and inducible NOS enzymatic activity, nitrosative status, and relative gene expression of neuronal and inducible NOS. Hippocampal constitutive NOS activity increased initially but was superseded by the inducible isoform as stress duration was prolonged. Interestingly, hippocampal neuronal NOS and interleukin-1β mRNA expression was downregulated, while the inducible NOS isoform was upregulated in conjunction with other inflammatory markers. This pro-inflammatory phenotype within the hippocampus was further confirmed with an increase in the glucocorticoid-antagonizing macrophage migration inhibitory factor, Mif, and the glial surveillance marker, Ciita. This indicates that despite high levels of glucocorticoids, acute stress sensitizes a neuroinflammatory response within the hippocampus involving both pro-inflammatory cytokines and inducible NOS while concurrently modulating the immunophenotype of glia. Furthermore, there was a delayed increase in striatal inducible NOS expression while no change was found in other pro-inflammatory mediators. This suggests that short term stress induces a generalized increase in inducible NOS signaling that coincides with regionally specific increased markers of adaptive immunity and inflammation within the brain.

1. Introduction

In response to a stressor, sympathetic activation results in the release of epinephrine and norepinephrine from the adrenal medulla, causing a catecholamine surge in the blood circulation, generating a 'fight-or-flight' response [23]. Subsequent activation of the hypothalamic–pituitary–adrenal (HPA) axis leads to an increase in the amplitude and synchronization of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) secretions from the parvocellular neuroendocrine cells [50]. Synergistically, AVP acts with CRH on the anterior pituitary corticotrophs, potentiating the release of adrenocorticotropic hormone (ACTH), which in turn stimulates adrenal glucocorticoid production. The stress-induced hyperactivity also activates the renin–angiotensin–aldosterone system which, via the action of angiotensin II on the paraventricular nucleus of the hypothalamus, facilitates stimulation of CRH, ACTH, and adrenal glucocorticoids [2,20,43,51]. The biologically available fractions of corticosterone not bound to high affinity carrier proteins are capable of crossing the blood–brain barrier and are responsible for mediating negative feedback [39]. This feedback mechanism depends on the binding of corticosteroids to cytosolic glucocorticoid receptors (GR) which translocate into the nucleus and interact as homodimers with specific glucocorticoid responsive elements, thus repressing Crh and Avp gene expression to terminate the stress response and restore homeostasis [54].

Following the release of the stress hormone corticosterone, blood glucose concentrations rapidly elevate through processes of hepatic gluconeogenesis and insulin resistance necessary for the adaptive responses to stress, including increased mitochondrial ATP production via oxidative phosphorylation [16,26,49]. This glucocorticoid-driven increase in cellular metabolism is subject to a small percentage of

Abbreviations: CIITA, class II major histocompatibility complex transactivator; GR, glucocorticoid receptor; MIF, macrophage migration inhibitory factor; sGC, soluble guanylyl cyclase

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electron 'leakage' from the electron transport chain, ultimately generating free radicals such as superoxide in place of water molecules, in addition to other members of the reduction/oxidation (redox) system such as nitric oxide (NO) [11,47]. We have previously demonstrated that peripheral measures of oxidative and nitrosative status are affected significantly by an acute psychological stress and can persist beyond the cessation of the stressor [12,46]. Recently, there has been considerable interest in elucidating the role of the redox system in the downstream cellular events initiated by stress exposure. Zhou and colleagues [59] found that the expression and activity of the constitutive enzyme, neuronal nitric oxide synthase (nNOS), which produces NO in central and peripheral neurons, increased following chronic mild stress in the hippocampus of adult mice. No change was observed in the constitutive endothelial isoform (endothelial nitric oxide synthase; eNOS) and the transcription-dependent inducible nitric oxide synthase (*i*NOS). However, Olivenza and colleagues [37], using a chronic immobilization paradigm, and Peng and colleagues [38], using unpredictable chronic mild stress, demonstrated that *i*NOS expression was increased in the cerebral cortex. The latter group suggested that this *i*NOS-derived NO following chronic stress may contribute to depressive-like behaviors in mice. A single episode of 6 h acute restraint stress in rats also significantly increased *i*NOS activity in the cerebral cortex, an effect mediated by nuclear factor Kappa-light-chain-enhancer of activated B cells (NF-κB) signaling [29,30].

A number of studies have now implicated NO at both the modulatory and mechanistic levels of the stress system. Mohn and colleagues [31] discovered that corticosterone is released via a NO and prostaglandin-dependent pathway stimulated by either ACTH or the NO donor, sodium nitroprusside, highlighting the importance of redox molecules in the neuroendocrine stress system under physiological conditions. Using a chronic mild stress paradigm, Zhou and colleagues [58] showed that stress increases hippocampal nNOS expression via a MR-dependent mechanism causing chronic production of NO, ultimately leading to down-regulation of GR, a key etiological factor in the development of depression. Together, these studies demonstrate that the neuronal isoform of NOS in limbic regions is predominately associated with chronic stress exposure, while *i*NOS in cortical regions is responsive to both acute and chronic stress. However, the cellular, physiological, and functional effects of the nitrergic system following short term stress are poorly understood. In this study, we used the hippocampus and striatum, regions that display differential stress sensitivity and redox responses, to characterize the temporal changes in the neuronal and inducible NOS isoforms and pro-inflammatory markers following acute psychological stress.

2. Materials and methods

2.1. Experimental animals

Outbred male Wistar rats (Rattus *norvegicus*) aged 6–7 weeks postnatal, weighing 297.9 ± 3.8 g were sourced from The University of Queensland Biological Resources breeding colony. Rats were housed individually under standard laboratory conditions (22 ± 2 °C; 55 ± 5% humidity) with a 12:12 h light–dark cycle (lights off at 12.30 h). Standard rat chow and water were available ad libitum. All experimental procedures were in accordance with regulations and policies outlined by The University of Queensland Animal Ethics Committee with AEC approval numbers 018/11.

2.2. Habituation and transportation

Rats were habituated to human handling for 10 min per day one week prior to experimentation. On each experimental day, rats were transported in individual home cages from the colony room to an experimental room within the same animal facility. Rats were acclimatized to the novel experimental room under low light and noise for one hour prior to treatment. To isolate the effects of restraint stress, all rats were deprived of food and water during the one hour habituation and treatment period.

2.3. Acute restraint stress

Rats were subjected to acute restraint stress using adjustable wire mesh restrainers to avoid overheating and body compression. The wire mesh restrainer was made of a PVC skeleton coated externally with welded wire mesh (6 mm × 6 mm grid). Acute restraint stress was applied within individual home cages (stress treatment starting at 13:30 h) enclosed with a larger ventilated containment chamber (68 cm (L) × 45 cm (W) × 38.5 cm (H)) to reduce any external disturbances.

2.4. Treatment protocol and tissue collection

Animals were randomly allocated to 0 (control), 60, 120, or 240 min stress treatment groups (n=5–8 per group). At the end of each treatment period, rats were weighed and sacrificed with sodium pentobarbital (100 mg/kg i.p. injection, Lethabarb, Virbac) and a single blood sample was collected via cardiac puncture into ice-chilled heparinized tubes (20 IU/mL blood). The brain was rapidly removed and frozen on powdered dry ice for storage at −80 °C. Whole blood was centrifuged at 200 × *g* for 5 min and supernatant plasma was collected and stored at −80 °C for later determination of corticosterone. Frozen brains were sectioned on a cryostat and the hippocampus and striatum were dissected out on a powdered dry ice-embedded metal platform according to the rat brain atlas [41]. Regionalized neural tissues were stored at −80 °C for later determination of the enzymatic activity of NOS, NO metabolism, 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) activation, and relative gene expression.

2.5. Plasma corticosterone assay

Corticosterone concentrations were determined using an in-house radioimmunoassay employing a highly specific ovine anti-rat corticosterone polyclonal antibody (Sapphire Bioscience Pty. Ltd.) and tritiated [1, 2, 6, 7-^{3}H]-corticosterone tracer as previously described in Spiers and colleagues [46]. Radioactivity was counted in liquid scintillation cocktail (Ultima Gold™, Perkin Elmer) using a Liquid Scintillation Spectrometer (Tri-Carb 3100 TR, Perkin Elmer). Concentrations of unknown sample were determined from a standard curve and corrected for dilution and extraction efficiency. Dichloromethane extraction recovery was 89.65% and intra- and inter-assay coefficients of variation were 4.70% and 4.59% respectively.

2.6. Neural tissue preparation

The hippocampus and striatum were homogenized (IKA® Ultra-Turrax® T10) in 20 volumes (w/v) of phosphate buffered saline (PBS; 50 mM, pH=7.4) and the crude homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting supernatant was used for the determination of NOS enzymatic activity, DAF-FM activation, and protein concentrations. For the quantification of total nitrite and nitrate (NO_x), 60 μL of the 20 volume supernatant was ultra-filtered through a 10-kDa molecular weight cut-off filter using commercially available centrifugal devices (Pall Nanosep®, Cheltenham, Australia).

2.7. Assay of calcium-independent and dependent nitric oxide synthase activity

The enzymatic activity of calcium-independent and dependent NOS isoform was determined by monitoring the conversion of