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Sleep Loss Mediates the Effect of Stress on Nitrergic Signaling in Female Mice

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Abstract

Nitric oxide (NO) has been implicated as an important neurotransmitter in stress responses and sleep regulatory processes. However, the role of NO in the relationship between stress and sleep remains unclear. The medial septum (MS) and vertical diagonal band (VDB), regions of the basal forebrain involved in sleep regulation, contain nitric oxide synthase (NOS) producing neurons. Additionally, NOS neurons in the dorsal raphe nucleus (DRN) encode information about stress duration. The role of nitrergic neurons in these regions in subserving sex-specific responses to stress and sleep loss has yet to be elucidated. In this study, NADPH-d, an index of NOS activity, was used to examine the effects of acute restraint stress and sleep loss on NOS activity in the MS, VDB, and DRN. We show that NOS activity in response to restraint stress, total sleep deprivation (TSD), and partial sleep restriction (PSR) differs based on sex and region. Initial analysis showed no effect of restraint stress or TSD on NOS activity in the basal forebrain. However, investigation of each sex separately revealed that restraint stress and TSD significantly decrease NOS activity in the MS of females, but not males. Interestingly, the difference in NOS activity between restraint stress and TSD in females was not significant. Furthermore, PSR was not sufficient to affect NOS activity in males or females. These data suggest that restraint stress and sleep loss regulate NOS activation in a sex-dependent manner, and that the NOS stress response in females may be mediated by sleep loss.

Keywords

nitric oxide synthase; basal forebrain; sleep; stress; mouse

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Author statement

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India Nichols: Conceptualization, Validation, Formal analysis, Investigation, Supervision, Project administration

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Introduction

There is a reciprocal relationship between stress and sleep [1,2,3]. Stress often produces sleep loss which, in turn, can worsen physiological stress responses. The mechanisms that underlie this complex interaction remain unclear. Nitric oxide (NO), a diffusible signaling molecule, plays a role in many physiological responses. While NO signaling is crucial throughout the body, such as in the cardiovascular [4] and gastrointestinal [5] systems, it also has a functional role in the central nervous system [6]. In mammals, stressful stimuli in the environment triggers nitric oxide synthase (NOS) activation in the brain [7], particularly in the dorsal raphe nucleus (DRN) of the brainstem [8], prefrontal cortex, hippocampus, and hypothalamus [9]. Thus, NO signaling serves as a critical mediator of stress responses.

NO signaling is also involved in sleep regulation [10,11]. In the basal forebrain, NO has been implicated in sleep homeostasis, the process which underlies the ability to recover from sleep loss. Sleep deprivation induces NO production in the basal forebrain [12], and NO production in this area is necessary for recovery sleep [13]. Specifically in the medial septum (MS) and vertical diagonal band (VDB) of the basal forebrain, NOS has been shown to localize in cholinergic neurons [14,15,16]. Due to the convergence of stress and sleep regulatory mechanisms on the nitrergic system, we hypothesized that restraint stress and sleep loss may result in similar downstream effects on NOS activity.

There are sex differences in susceptibility to stress and sleep disturbances [17,18,19,20]. For instance, females are more resilient to chronic stress [21], and acute stress [22] than males. Also, there are sex differences in the risk for stress-sensitive psychological disorders, such as post-traumatic stress disorder and depression [23,24]. Furthermore, the ability to recover from sleep loss is partially dependent on sex. Females exhibit a more robust recovery response than males following sleep loss [25], and this sex difference is driven by gonadal hormones [25,26] and sex chromosome complement [27]. However, the mechanisms that establish sex differences in the interaction between stress and sleep regulation are not well understood.

In this study, we examined the relationship between stress-induced and sleep loss-induced NOS activity, and whether sex-specific responses to acute stress and sleep loss are reflected in distinct changes in NOS activity. In order to investigate this, we exposed mice to one of three conditions: a) restraint stress, b) total sleep deprivation (TSD), or c) partial sleep restriction (PSR), and NOS activity was measured using a NADPH-d stain following these interventions.

Material and Methods

Animals

Male and female adult C57BL/6J mice (Jackson Laboratory) were maintained on a 12h:12h light/dark cycle. Food and water were provided *ad libitum.* Mice were group housed in a standard cage (4/cage) before being randomly assigned to groups. This study used a total of 66 mice divided into 2 cohorts. The first cohort of male (n=18) and female (n=18) mice were

used to determine and compare the effects of restraint stress and TSD. The second cohort of male (n=14) and female (n=16) mice were used to determine the effects of PSR.

Restraint stress

Mice were separately held in semi-cylindrical, plastic restraint devices while breathing and movement were closely monitored [28]. Restraint devices were placed in the animal's home cage. Criteria for removal from the device were abnormal breathing, lesions, illnesses, or other irregular behavioral changes. No animals needed to be removed. Control mice were maintained in their home cage with *ad libitum* access to food and water. Mice were restrained for 6h starting at the onset of the light phase in a 12h:12h light/dark cycle, and were sacrificed immediately following restraint.

Total sleep deprivation (TSD)

Mice underwent 6h of TSD using a gentle-handling protocol, which includes cage tapping, introduction of novel objects, and gentle touching when mice displayed signs of sleep onset [29]. TSD began at the onset of the light phase in a 12h:12h light/dark cycle. Control mice were left undisturbed with *ad libitum* access to food and water. Mice were sacrificed immediately following TSD.

Partial sleep restriction (PSR)

Mice habituated to slowly rotating wheels for 1 week in a 12h:12h light/dark cycle. Following habituation, wheels began rotating at the onset of the light phase at 1 revolution/min for 24h [29]. Food and water were provided *ad libitum* while mice were in wheels. Control mice were maintained in their home cage with *ad libitum* access to food and water. Animals were sacrificed immediately following PSR.

Corticosterone extraction

To assess whether restraint stress or TSD induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, fecal corticosterone (CORT) levels were measured. CORT was extracted from the feces using a corticosterone competitive ELISA kit (ThermoFisher Scientific).

Tissue collection

Animals were anesthetized with sodium pentobarbital administered at a concentration of 150 mg/kg to each mouse. Animals were perfused transcardially with phosphate buffered solution (PBS), followed by 10% formalin. Brains were collected and placed to post-fix in formalin overnight at 4 ° C. Following fixation, brains were placed in 30% sucrose at 4 ° C until sectioning. Coronal sections from 1.34 mm to 0.38 mm relative to bregma containing the basal forebrain regions of interest (MS and VDB) were sectioned at 40 μm on a cryostat. Coronal sections from −4.66 mm to −5.32 mm relative to bregma containing the DRN were sectioned at 20 μm. Sections were collected based on figures from the mouse brain atlas [30]. Following collection, sections were placed in PBS and stored at 4 ° C. Four brains were damaged during the tissue collection stage (2 controls, 1 restraint stress, 1 TSD), and were removed from analysis.

NADPH-d staining

Free floating sections were placed in a 24-well plate containing PBS with tween at room temperature for 30 min to allow for membrane permeability. A NADPH-diaphorase (NADPH-d) stain was used to label NOS+ cells. In the presence of NADPH, NOS reduces tetrazolium dyes, such as nitroblue, to a dark blue formazan product. NADPH-d staining solution was made using 200 μL of nitroblue, 400 μL of NADPH TS, 60 μL of Triton X-100, and 20 mL of PBS. The PBST was aspirated out of each well, and 500 μL of NADPH-d solution was applied to each well. Wells were covered in aluminum foil, and allowed to incubate at 37 ° C for 1h. Once sections were stained dark purple after 1h, sections were held in wells containing PBS at 4 ° C until mounting [31]. Tissue sections were mounted onto chromealum gelatin-coated slides, dried, and coverslipped.

Imaging

The mouse brain atlas [30] was used to define anatomical areas of interest. Every tenth section from 1.34 mm to 0.38 mm relative to bregma for the basal forebrain, and every tenth section from −4.66 mm to −5.32 mm relative to bregma for the DRN was stained and used for analysis. Images were acquired using a 20X objective lens on an EVOS FL AUTO light microscope. Lower magnification images were acquired on a Zeiss Stereomicroscope (Stemi SV 11 Apo) equipped with an Axiocam using the AxioVision software (Carl Zeiss).

NOS measurement

All images were converted from RGB color to gray scale for analysis using ImageJ. 2–5 sections for each animal were used to measure NADPH-d optical density and cell count. All analysis was performed by a single observer. Optical density was obtained by measuring the mean gray pixel value from representative cells in the MS, VDB, and DRN using ImageJ. A calibrated optical density curve was used to obtain the optical density value. The mean gray value is the sum of the gray values of all pixels in the selection divided by the number of pixels [8].

Statistical analysis

Statistical analysis was performed using SPSS software. To determine the effect of restraint stress and TSD in the MS, VDB, and DRN, a two-way analysis of variance (ANOVA) was performed for each region with sex and condition as factors. The Tukey *post hoc* test was used when appropriate. Percentage change from control was calculated using GraphPad Prism 8. In order to determine the effect of PSR in the MS and VDB, a two-way ANOVA was performed for each region using sex and condition as factors. To determine changes in CORT levels, a two-way ANOVA was performed with sex and condition as factors. The Tukey *post hoc* test was used when appropriate.

Results

Restraint stress and total sleep deprivation (TSD) significantly decrease NOS activity in the medial septum (MS) of females

First, we examined the effects of restraint stress and TSD on NOS activity in the MS, VDB, and DRN. NADPH-d optical density was used as a measure of NOS activity. A two-way ANOVA was performed for each brain region. In the MS, there was a significant interaction between sex and condition. This suggests that the ability of restraint stress or TSD to alter NADPH-d optical density is sex-dependent. A *post hoc* analysis revealed a significant difference in NADPH-d optical density between control and restraint stress conditions, and between control and TSD conditions in females. However, there was no significant difference between restraint stress and TSD conditions. There were no significant differences between conditions in males (Fig. 1A, C; Table 1). In the VDB, there was no effect of sex or condition, and no significant interaction between the factors (Fig. 1B, D; Table 1). In the DRN, there was no effect of sex or condition, and no significant interaction between the factors (Table 1).

Partial sleep restriction (PSR) has no effect on NOS activity in the medial septum (MS) and vertical diagonal band (VDB)

Next, we examined the effect of PSR in a slowly-rotating wheel on NOS activity in the MS and VDB. A two-way ANOVA revealed a significant effect of sex. However, there was no effect of condition, and no significant interaction between the factors (Fig. 2; Table 1). These results suggest that PSR has no effect on NOS activity.

Total sleep deprivation (TSD) via gentle-handling does not elevate corticosterone levels

A hypothalamic-pituitary-adrenal (HPA) axis response to stress includes increased corticosterone (CORT) levels [32,33]. Fecal CORT measurements are non-invasive, reliably reflect circulating CORT levels, and act as a biomarker of stress responses [34,35]. In order to ensure that gentle-handling during TSD did not induce a HPA stress response, we examined CORT levels in feces following the intervention. A two-way ANOVA revealed a significant difference in CORT levels between conditions ($F(2,17)=14.201$; $p=0.001$), no difference between sex ($F(1,17)=0.402$; $p=0.538$), and no signicant interaction between the two factors ($F(2,17)=0.469$; $p=0.637$). *Post hoc* tests showed significantly elevated CORT levels in restrained mice as compared to controls ($p=0.002$) and as compared to TSD mice ($p=0.002$). However, there were no significant changes in CORT levels in TSD mice ($p>0.05$) as compared to controls. This suggests that TSD does not activate a HPA stress response resulting in CORT release.

Discussion

Sex differences in physiological and behavioral responses to acute stress have been frequently reported [36,37]. However, the mechanisms mediating these sex differences in susceptibility to stress are unclear. In this study, we show a significant decrease in NOS activity in females following restraint stress and TSD, with no significant difference between the restraint stress and TSD groups. This finding suggests that sleep loss which occurs

during the restraint stress episode may contribute to the effect of restraint stress on NOS activity, rather than solely a consequence of stress mechanisms. Thus, a sleep regulatory mechanism, mediated by NOS, may encode the stress responses in females but not males.

Interestingly, NOS activity is not affected by PSR. During PSR in the slowly rotating wheel, animals find opportunities for microsleep that increases with duration in the wheel, and thus dissipates some sleep pressure during that period [38]. Therefore, it appears that acute TSD is required to affect NOS activity in the MS, and PSR in a slowly-rotating wheel is not sufficient.

NOS activity in the DRN of rats has been shown to respond dynamically to restraint stress [8]. In order to determine if the NOS response in rats could be recapitulated in mice, we examined the effect of restraint stress and TSD on NOS activation in the DRN of mice. Since the mouse DRN does not contain NOS in the midline, our study focused on the lateral DRN [39]. We show that restraint stress and TSD have no effect on NOS activity in the lateral DRN of mice, which suggests that nitrergic neurons in the DRN of mice do not process stress information in the same way they do in rats.

NO has been shown to stimulate the release of adenosine in the basal forebrain [40]. During periods of wakefulness, extracellular adenosine accumulates in the basal forebrain, where it acts to inhibit wake-promoting neurons and activate sleep-promoting neurons [41]. However, blocking adenosine alpha-1 (A1) receptors do not completely mitigate NO action, which suggests that NO may have effects on sleep regulatory mechanisms independent of adenosine [42]. Previous work has shown that NO levels increase in the basal forebrain during sleep deprivation, thus suggesting its role as a sleep-promoting factor [13]; however, this study only examined males. While our study examined NOS activation as a proxy for NO levels, our results suggest that NO levels may be differentially affected by TSD depending on sex. We show that NOS activity is significantly decreased in females following TSD, which may imply that NOS in the MS of females does not carry a sleep-promoting function. Also, the difference in NOS activation between TSD and restraint stress in females is not significant. This finding suggests that NOS activity in response to sleep loss may act as a mediator of stress responses in females, rather than functioning as a somnogen. Our results add to the literature on sex differences in NO action, which have also been shown in motor coordination [43], and affective behaviors [44].

The NADPH-diaphorase (NADPH-d) assay is a well-established method to localize and measure activity-dependent changes in NOS producing neurons. A diaphorase defines an enzyme that can oxidize a reduced form of coenzyme NAD, for example, NADPH, which is a reduced form of NADP+. It has been shown that NADPH-d is a NOS [45]; thus, the intensity of NADPH-d staining provides an accurate measure of NOS activity [46,47,48]. Furthermore, NADPH-d staining has been used to show that NOS colocalizes in magnocellular cholinergic neurons in the MS and VDB of the rat basal forebrain [16]. Therefore, the NOS reported in our findings may be colocalizing with cholinergic neurons of the MS.

In order to validate that TSD via gentle-handling does not induce a HPA stress response, CORT levels were measured. There was a significant elevation in CORT levels in restrained mice as compared to controls, which corroborates previous literature [28]. However, there was no significant difference in CORT levels between control and TSD mice. This suggests that TSD via gentle-handling does not induce a HPA stress response. While there are other stress responses that do not depend on HPA overstimulation, due to the role of the basal forebrain as a sleep regulatory center, it is likely that this change in NOS activity is mediated by sleep homeostasis. While CORT levels were not measured in the PSR group, it has been shown that PSR does increase CORT levels [49, 50]. Regardless of the elevation of CORT levels, there was no effect of PSR on NOS activity due to the insufficient sleep loss that occurs during PSR. Thus, this supports our finding that a HPA stress response is not responsible for the change in NOS activation in the MS.

In summary, these data show that the ability of acute restraint stress to decrease NOS activity in the MS of females is dependent on sleep loss. Therefore, this stress response may be encoded by a sleep regulatory mechanism. The sex-dependent regulation of NOS activity in the basal forebrain may underlie key sex differences in stress and sleep regulatory systems.

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References

[1]. Olini N, Rothfuchs I, Azzinnari D, Pryce CR, Kurth S, Huber R, 2017. Chronic social stress leads to altered sleep homeostasis in mice. Behavioural brain research, 327, 167–173. [PubMed: 28315757]

[2]. Kaushik ML, Aritake K, Takeuchi A, Yanagisawa M, Urade Y, 2017. Octacosanol restores stress-affected sleep in mice by alleviating stress. Scientific reports, 7(1), 8892. [PubMed: 28827687]

[3]. I Almojali A, Almalki SA, Alothman AS, Masuadi EM, K Alaqeel M, 2017. The prevalence and association of stress with sleep quality among medical students. Journal of epidemiology and global health, 7(3), 169–174. [PubMed: 28756825]

[4]. Farah C, Michel L, Balligand JL, 2018. Nitric oxide signalling in cardiovascular health and disease. Nature reviews. Cardiology, 15(5), 292–316. [PubMed: 29388567]

[5]. de Oliveira GA, Cheng R, Ridnour LA, Basudhar D, Somasundaram V, McVicar DW, Monteiro HP, Wink DA, 2017. Inducible Nitric Oxide Synthase in the Carcinogenesis of Gastrointestinal Cancers. Antioxidants & redox signaling, 26(18), 1059–1077. [PubMed: 27494631]

[6]. Vincent SR, 2010. Nitric oxide neurons and neurotransmission. Progress in neurobiology, 90(2), 246–255. [PubMed: 19853011]

[7]. Kumar A, Chanana P, 2017. Role of Nitric Oxide in Stress-Induced Anxiety: From Pathophysiology to Therapeutic Target. Vitamins and hormones, 103, 147–167. [PubMed: 28061969]

[8]. Nichols IS, I Jones M, Okere C, Ananaba G, Bush B, Gray C, Brager A, Ehlen JC, Paul K, 2017. Nitrergic neurons of the dorsal raphe nucleus encode information about stress duration. PloS one, 12(11), e0187071.

[9]. Gądek-Michalska A, Tadeusz J, Bugajski A, Bugajski J, 2019. Chronic Isolation Stress Affects Subsequent Crowding Stress-Induced Brain Nitric Oxide Synthase (NOS) Isoforms and

Hypothalamic-Pituitary-Adrenal (HPA) Axis Responses. Neurotoxicity research, 36(3), 523–539. [PubMed: 31209786]

[10]. Williams JA, Vincent SR, Reiner PB, 1997. Nitric oxide production in rat thalamus changes with behavioral state, local depolarization, and brainstem stimulation. The Journal of neuroscience : the official journal of the Society for Neuroscience, 17(1), 420–427. [PubMed: 8987767]

[11]. Hars B, 1999. Endogenous nitric oxide in the rat pons promotes sleep. Brain research, 816(1), 209–219. [PubMed: 9878741]

[12]. Kalinchuk AV, McCarley RW, Porkka-Heiskanen T, Basheer R, 2010. Sleep deprivation triggers inducible nitric oxide-dependent nitric oxide production in wakeactive basal forebrain neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience, 30(40), 13254–13264.

[13]. Kalinchuk AV, Lu Y, Stenberg D, Rosenberg PA, Porkka-Heiskanen T, 2006. Nitric oxide production in the basal forebrain is required for recovery sleep. Journal of neurochemistry, 99(2), 483–498. [PubMed: 17029601]

[14]. Vincent SR, Kimura H, 1992. Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience, 46(4), 755–784. [PubMed: 1371855]

[15]. Pasqualotto BA, Vincent SR, 1991. Galanin and NADPH-diaphorase coexistence in cholinergic neurons of the rat basal forebrain. Brain research, 551(1–2), 78–86. [PubMed: 1717107]

[16]. Kitchener PD, Diamond J, 1993. Distribution and colocalization of choline acetyltransferase immunoreactivity and NADPH diaphorase reactivity in neurons within the medial septum and diagonal band of Broca in the rat basal forebrain. The Journal of comparative neurology, 335(1), 1–15. [PubMed: 8408771]

[17]. Dolsen MR, Crosswell AD, Prather AA, 2019. Links Between Stress, Sleep, and Inflammation: Are there Sex Differences?. Current psychiatry reports, 21(2), 8. [PubMed: 30729328]

[18]. Hajali V, Andersen ML, Negah SS, Sheibani V, 2019. Sex differences in sleep and sleep loss-induced cognitive deficits: The influence of gonadal hormones. Hormones and behavior, 108, 50–61. [PubMed: 30597139]

[19]. Goldstein-Piekarski AN, Greer SM, Saletin JM, Harvey AG, Williams LM, P Walker M, 2018. Sex, Sleep Deprivation, and the Anxious Brain. Journal of cognitive neuroscience, 30(4), 565–578. [PubMed: 29244642]

[20]. Bangasser DA, Wicks B, 2017. Sex-specific mechanisms for responding to stress. Journal of neuroscience research, 95(1–2), 75–82. [PubMed: 27870416]

[21]. Peay DN, Saribekyan HM, Parada PA, Hanson EM, Badaruddin BS, M Judd J, Donnay ME, Padilla-Garcia D, Conrad CD, 2020. Chronic unpredictable intermittent restraint stress disrupts spatial memory in male, but not female rats. Behavioural brain research, 383, 112519.

[22]. Conrad CD, Jackson JL, Wieczorek L, Baran SE, Harman JS, Wright RL, Korol DL, 2004. Acute stress impairs spatial memory in male but not female rats: influence of estrous cycle. Pharmacology, biochemistry, and behavior, 78(3), 569–579. [PubMed: 15251266]

[23]. Kessler RC, Petukhova M, Sampson NA, Zaslavsky AM, Wittchen H, 2012. Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. International journal of methods in psychiatric research, 21(3), 169–184. [PubMed: 22865617]

[24]. Ferrari AJ, Somerville AJ, Baxter AJ, Norman R, Patten SB, Vos T, Whiteford HA, 2013. Global variation in the prevalence and incidence of major depressive disorder: a systematic review of the epidemiological literature. Psychological medicine, 43(3), 471–481. [PubMed: 22831756]

[25]. Paul KN, Dugovic C, Turek FW, Laposky AD, 2006. Diurnal sex differences in the sleep-wake cycle of mice are dependent on gonadal function. Sleep, 29(9), 1211–1223. [PubMed: 17040009]

[26]. Deurveilher S, Rusak B, Semba K, 2009. Estradiol and progesterone modulate spontaneous sleep patterns and recovery from sleep deprivation in ovariectomized rats. Sleep, 32(7), 865–877. [PubMed: 19639749]

[27]. Ehlen JC, Hesse S, Pinckney L, Paul KN, 2013. Sex chromosomes regulate nighttime sleep propensity during recovery from sleep loss in mice. PloS one, 8(5), e62205.

[28]. Buynitsky T, Mostofsky DI, 2009. Restraint stress in biobehavioral research: Recent developments. Neuroscience and biobehavioral reviews, 33(7), 1089–1098. [PubMed: 19463853]

[29]. Colavito V, Fabene PF, Grassi-Zucconi G, Pifferi F, Lamberty Y, Bentivoglio M, Bertini G, 2013. Experimental sleep deprivation as a tool to test memory deficits in rodents. Frontiers in systems neuroscience, 7, 106. [PubMed: 24379759]

[30]. Franklin K, Paxinos G, 2008. The Mouse Brain in Stereotaxic Coordinates. Academic Press, Inc.

[31]. Gonzalez-Zulueta M, Dawson VL, Dawson TM, 2001. Histochemical analysis of nitric oxide synthase by NADPH diaphorase staining. Current protocols in toxicology, Chapter 10.

[32]. Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, Scheimann J, Myers B, 2016. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. Comprehensive Physiology, 6(2), 603–621. [PubMed: 27065163]

[33]. Nohara M, Tohei A, Sato T, Amao H, 2016. Evaluation of response to restraint stress by salivary corticosterone levels in adult male mice. The Journal of veterinary medical science, 78(5), 775–780. [PubMed: 26852731]

[34]. Thanos PK, Cavigelli SA, Michaelides M, Olvet DM, Patel U, Diep MN, Volkow ND, 2009. A non-invasive method for detecting the metabolic stress response in rodents: characterization and disruption of the circadian corticosterone rhythm. Physiological research, 58(2), 219–228. [PubMed: 18380537]

[35]. Cinque C, Zinni M, Zuena AR, Giuli C, Alemà SG, Catalani A, Casolini P, Cozzolino R, 2018. Faecal corticosterone metabolite assessment in socially housed male and female Wistar rats. Endocrine connections, 7(2), 250–257. [PubMed: 29301863]

[36]. Luine V, Gomez J, Beck K, Bowman R, 2017. Sex differences in chronic stress effects on cognition in rodents. Pharmacology, biochemistry, and behavior, 152, 13–19. [PubMed: 27566290]

[37]. McDowell AL, Fransen KM, Elliott KS, Elghouche A, Kostylev PV, O'Dea PK, Garraghty PE, 2015. Sex Differences and the Impact of Chronic Stress and Recovery on Instrumental Learning. Neuroscience journal, 2015, 697659.

[38]. Ehlen JC, Jefferson F, Brager AJ, Benveniste M, Paul KN, 2013. Periodamplitude analysis reveals wake-dependent changes in the electroencephalogram during sleep deprivation. Sleep, 36(11), 1723–1735. [PubMed: 24179307]

[39]. Fu W, Le Maître E, Fabre V, Bernard JF, David Xu ZQ, Hökfelt T, 2010. Chemical neuroanatomy of the dorsal raphe nucleus and adjacent structures of the mouse brain. The Journal of comparative neurology, 518(17), 3464–3494. [PubMed: 20589909]

[40]. Rosenberg PA, Li Y, Le M, Zhang Y, 2000. Nitric oxide-stimulated increase in extracellular adenosine accumulation in rat forebrain neurons in culture is associated with ATP hydrolysis and inhibition of adenosine kinase activity. The Journal of neuroscience : the official journal of the Society for Neuroscience, 20(16), 6294–6301. [PubMed: 10934281]

[41]. Scammell TE, Arrigoni E, Lipton JO, 2017. Neural Circuitry of Wakefulness and Sleep. Neuron, 93(4), 747–765. [PubMed: 28231463]

[42]. Kostin A, Stenberg D, Kalinchuk AV, Porkka-Heiskanen T, 2008. Nitric oxide modulates the discharge rate of basal forebrain neurons. Psychopharmacology, 201(1), 147–160. [PubMed: 18661122]

[43]. Muñoz-Castañeda R, Díaz D, Avila-Zarza CA, Alonso JR, Weruaga E, 2014. Sex-influence of nicotine and nitric oxide on motor coordination and anxiety-related neurophysiological responses. Psychopharmacology, 231(4), 695–706. [PubMed: 24081550]

[44]. Hu Y, Wu DL, Luo CX, Zhu LJ, Zhang J, Wu HY, Zhu DY, 2012. Hippocampal nitric oxide contributes to sex difference in affective behaviors. Proceedings of the National Academy of Sciences of the United States of America, 109(35), 14224–14229. [PubMed: 22891311]

[45]. Hope BT, Michael GJ, Knigge KM, Vincent SR, 1991. Neuronal NADPH diaphorase is a nitric oxide synthase. Proceedings of the National Academy of Sciences of the United States of America, 88(7), 2811–2814. [PubMed: 1707173]

[46]. Matsumoto T, Nakane M, Pollock JS, Kuk JE, Förstermann U, 1993. A correlation between soluble brain nitric oxide synthase and NADPH-diaphorase activity is only seen after exposure of the tissue to fixative. Neuroscience letters, 155(1), 61–64. [PubMed: 7689718]

[47]. Morris BJ, Simpson CS, Mundell S, Maceachern K, M Johnston H, Nolan AM, 1997. Dynamic changes in NADPH-diaphorase staining reflect activity of nitric oxide synthase: evidence for

a dopaminergic regulation of striatal nitric oxide release. Neuropharmacology, 36(11–12), 1589–1599. [PubMed: 9517430]

[48]. Okere CO, Waterhouse BD, 2006. Acute capsaicin injection increases nicotinamide adenine dinucleotide phosphate diaphorase staining independent of Fos activation in the rat dorsolateral periaqueductal gray. Neuroscience letters, 404(3), 288–293. [PubMed: 16835009]

[49]. Dumaine JE, Ashley NT, 2015. Acute sleep fragmentation induces tissue-specific changes in cytokine gene expression and increases serum corticosterone concentration. American journal of physiology. Regulatory, integrative and comparative physiology, 308(12), R1062–R1069. [PubMed: 25876653]

[50]. Sgoifo A, Buwalda B, Roos M, Costoli T, Merati G, Meerlo P, 2006. Effects of sleep deprivation on cardiac autonomic and pituitary-adrenocortical stress reactivity in rats. Psychoneuroendocrinology, 31(2), 197–208. [PubMed: 16154708]

Highlights

- Sleep loss and restraint stress affect NOS activity in the basal forebrain
- NOS activity decreases following sleep loss and restraint stress in females
- The effect of stress on NOS activity in females results from sleep loss

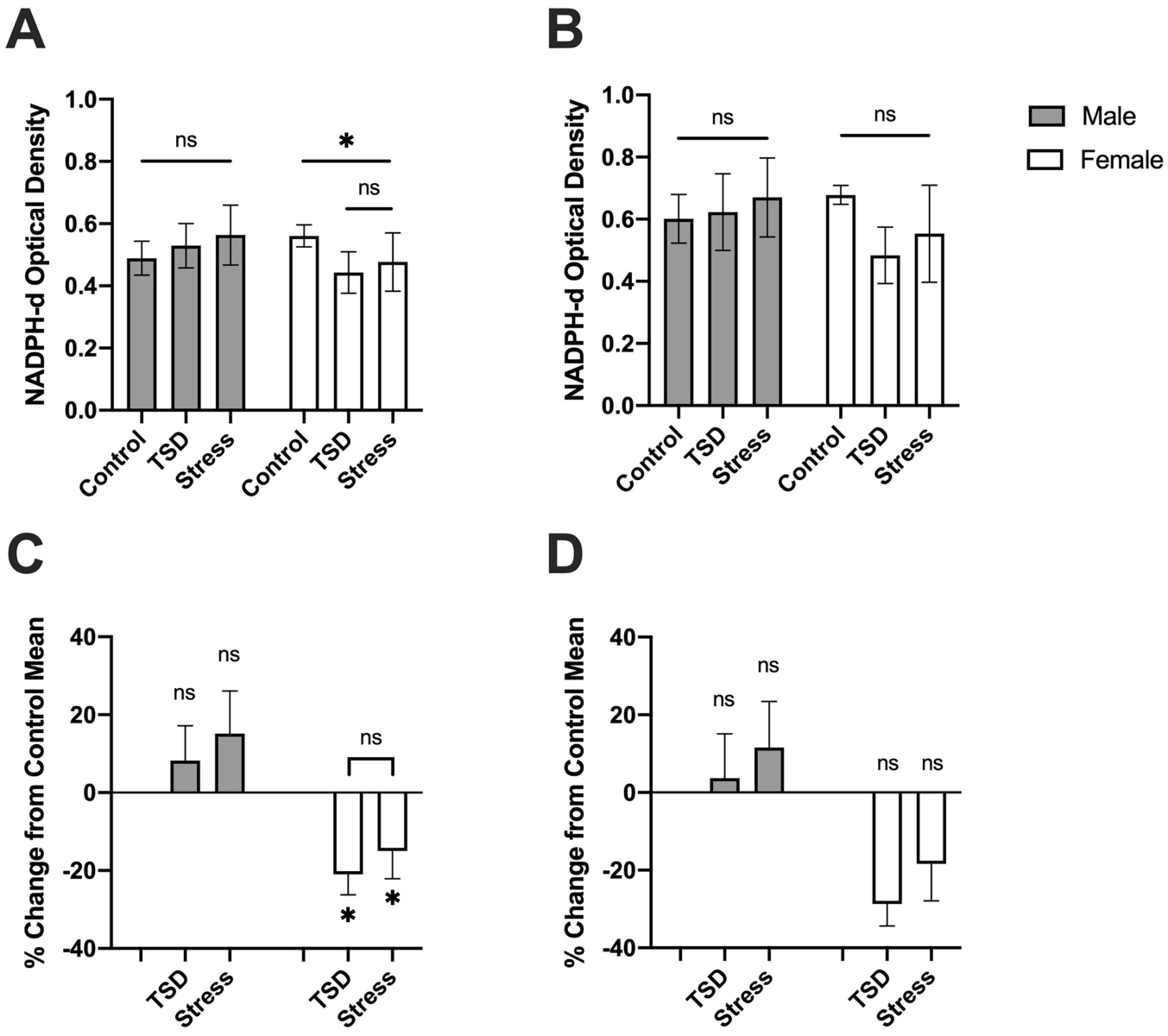

Figure 1. Effect of total sleep deprivation (TSD) and restraint stress on NOS activity in the basal forebrain

The NADPH-d optical density, a measure of NOS activity, is shown in the **(A)** medial septum (MS) and **(B)** vertical diagonal band (VDB). The percent change from control mean NADPH-d optical density in the **(C)** MS and **(D)** VDB are shown following TSD or restraint stress. Data are presented as mean ± SEM. * $p<0.05$ compared between condition (Tukey *post hoc* test).

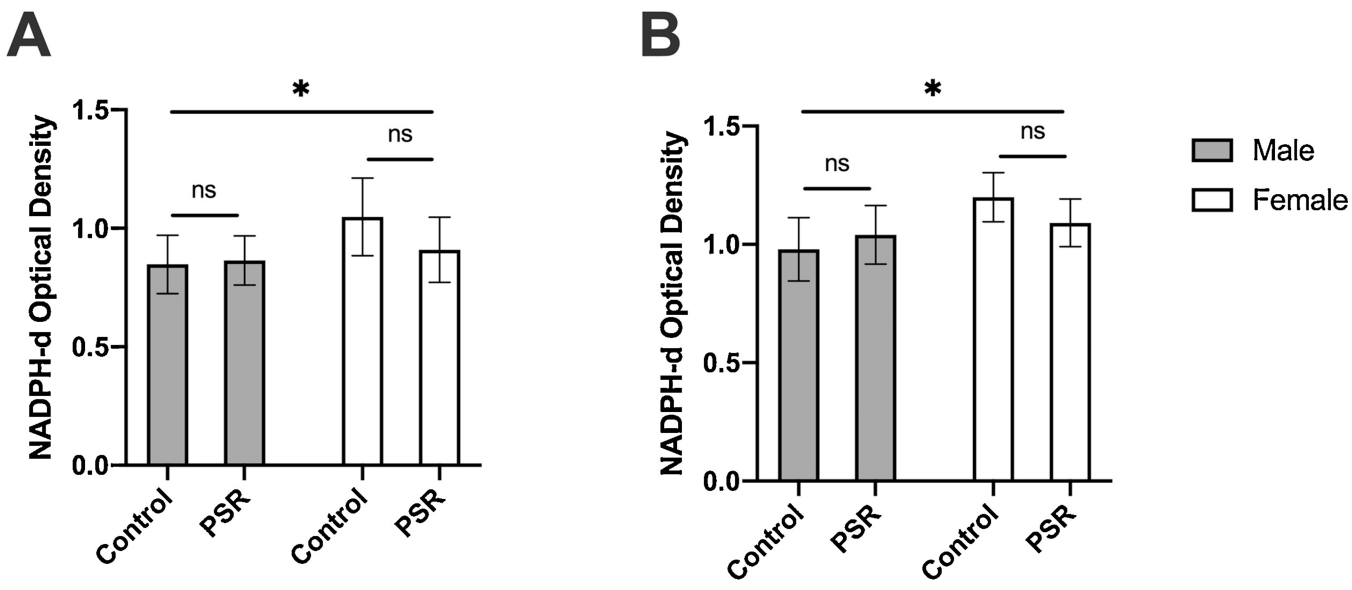

Figure 2. Partial sleep restriction (PSR) has no effect on NOS activity in the basal forebrain

The NADPH-d optical density, a measure of NOS activity, is shown in the **(A)** medial septum (MS) and **(B)** vertical diagonal band (VDB) under control or PSR conditions. Data are presented as mean ± SEM. * $p<0.05$ compared between sex (two-way ANOVA).

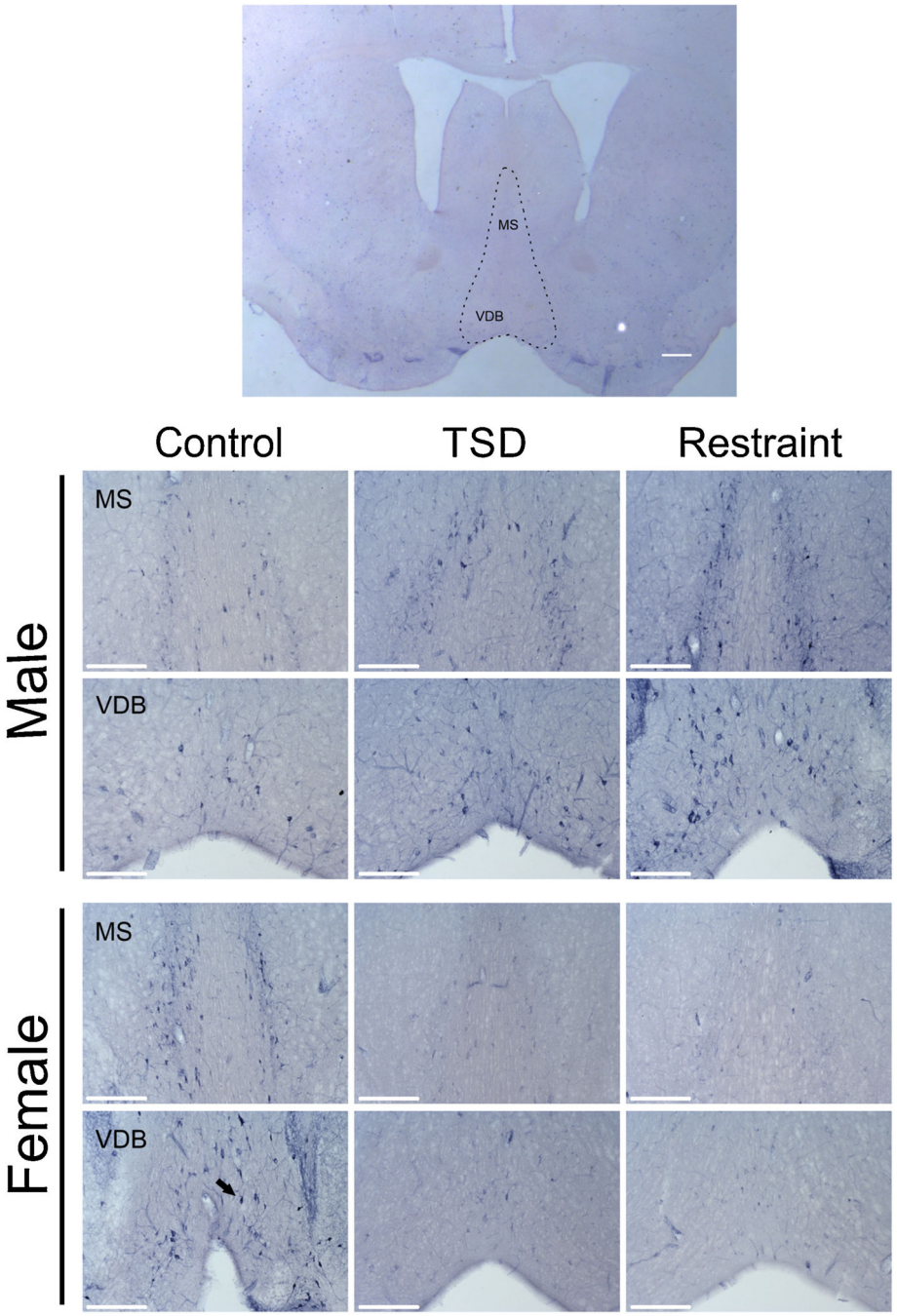

Figure 3. NADPH-diaphorase staining in the medial septum (MS) and vertical diagonal band (VDB)

A NADPH-diaphorase (NADPH-d) stain was used to measure NOS activity in the MS and VDB under control, TSD, and restraint stress conditions. Both TSD and restraint stress significantly decrease NOS activity in the MS of females. Arrowhead indicates a representative cell. Scale bar size 200μm in all figures.

Table 1.

Restraint stress and total sleep deprivation (TSD) affect NOS activity in females

Region	Sex	Condition (Control vs. Stress vs. TSD)	Interaction (Sex * Condition)	Tukey Multiple Comparisons		
MS	F(1,26)=1.673; p=0.207	F(2,26)=0.876; p=0.428	**F(2,26)=3.893; p=0.033**	Male	Control vs. Stress	p=0.361
					Control vs. TSD	p=0.726
					Stress vs. TSD	p=0.773
				Female	Control vs. Stress	**p=0.032**
					Control vs. TSD	**p=0.011**
					Stress vs. TSD	p=0.793
VDB	F(1,26)=2.321; p=0.140	F(2,26)=1.686; p=0.205	F(2,26)=2.964; p=0.069			
DRN	F(1,26)=0.811; p=0.376	F(2,26)=0.785; p=0.467	F(2,26)=0.822; p=0.451			

Region	Sex	Condition (Control vs. PSR)	Interaction (Sex * Condition)
MS	**F(1,26)=6.128; p=0.020**	F(1,26)=1.513; p=0.230	F(1,26)=2.437; p=0.131
VDB	**F(1,23)=9.214; p=0.006**	F(1,23)=0.279; p=0.603	F(1,23)=3.624; p=0.070

Data from each region were analyzed with a two-way ANOVA using sex and condition as factors. The Tukey *post hoc* test was used when appropriate. p values <0.05 were considered significant and are shown in bold.