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Plasma corticosterone, epinephrine, and norepinephrine levels increase during administration of nitrous oxide in rats

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Abstract

Nitrous oxide (N₂O) is a gaseous drug with abuse potential. Despite its common clinical use, little is known about whether N₂O administration activates the HPA axis and/or the sympathetic adrenomedullary system. The goal of this study was to determine whether 60% N₂O alters plasma concentrations of corticosterone (CORT), epinephrine (EPI), and norepinephrine (NE) in male Long-Evans rats. A gas-tight swivel assembly in the lid of a gas administration chamber allowed the remote collection of blood samples from an indwelling jugular vein catheter at four time-points: baseline and at 30, 60, and 120 min during a two-hour administration of 60% N₂O. Relative to baseline, plasma CORT (n=9) was significantly elevated at all three time-points during N₂O inhalation (mixed model analysis, p=0.001), and plasma EPI and NE levels were each significantly elevated (n=8, p = 0.001) at the 30-min assessment. EPI then declined and did not differ from baseline at the 60-min and 120-min assessments (p>0.05) whereas NE remained elevated (120-min, p=0.001). Administration of 60% N₂O increases circulating CORT, EPI, and NE, supporting N₂O as a physiological stressor. An N₂O-induced increase in CORT is consistent with the observation that addictive drugs typically activate the HPA axis causing increased plasma levels of glucocorticoids. Allostatic models of drug addiction typically involve stress systems and the possible role of stress hormones in N₂O-induced allostatic dysregulation is discussed.

Lay Summary:

Despite its common clinical use and abuse potential, little is known about nitrous oxide's effect on circulating stress hormones. This study found that inhalation of 60% nitrous oxide increases circulating levels of stress hormones in rats. This work will facilitate further research on the actions of nitrous oxide as a physiological stressor with relevance to its clinical use and addictive potential.

Keywords

stress; inhalant; addiction; allostasis; corticosterone; catecholamines

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Introduction

Nitrous oxide (N₂O) is a gaseous drug with a long history of clinical use in medicine and dentistry (Becker & Rosenberg, 2008; Sun et al., 2015). N₂O's low anesthetic potency limits its use as a sole anesthetic agent, although it is often administered in combination with other drugs as part of a balanced anesthetic approach (Sun et al., 2015). N₂O is also administered in clinical situations where achieving anesthesia is not the goal. The anxiolytic and modest analgesic effects of subanesthetic N₂O concentrations, when administered as 'N₂O - oxygen sedation,' can benefit many conscious patients undergoing painful or anxiety-provoking medical and dental procedures (Donaldson et al., 2012). N₂O is also an inhalant drug of abuse (Balster, 1998; Coussaert et al., 2013; Karr et al., 2016; Layzer, 1985; van Amsterdam et al., 2015). N₂O has positively reinforcing effects for some humans (Kangas & Walker, 2008; Walker & Zacny, 2001; Walker & Zacny, 2002); is self-administered by monkeys (Grubman & Woods, 1981; Wood et al., 1977) and rats (Ramsay et al., 2003; Ramsay et al., 2015); and increases dopamine in the nucleus accumbens of rats (Sakamoto et al., 2006). The adverse consequences from chronic N₂O abuse were recently reviewed (Garakani et al., 2016). Reviews of N₂O's pharmacological mechanisms of action are also available (Emmanouil & Quock, 2007; Sanders et al., 2008).

Many drugs of abuse activate the hypothalamic-pituitary-adrenal (HPA) axis (Armario, 2010; Stephens & Wand, 2012) and numerous reviews address the association between stress and addiction (e.g., Koob et al., 2014; Lemieux & al Absi, 2016; Piazza & Deroche-Gamonet, 2013). However, little is known about whether N₂O activates the HPA axis (e.g., increased plasma levels of glucocorticoids) and/or the sympathetic adrenomedullary (SAM) system (e.g., increased plasma levels epinephrine (EPI) and norepinephrine (NE)). Brandt et al. (1988) reported that N₂O markedly increased plasma cortisol in conscious human volunteers, and they suggested that this might result from N₂O-induced excitation or emotional stress. In contrast, Gillman et al. (1988) reported that N₂O did not significantly increase plasma cortisol, although there was a significant reduction from baseline levels 10 minutes after the 60-min N₂O administration had ended. However, those subjects received different N₂O concentrations (range 33%-60% N₂O, mean = 48.8% N₂O) that were individually titrated to produce 'subjective feelings of "floating" and relaxation' (p. 751). Fisher et al. (1987) administered 80% N₂O for 60 minutes to male Sprague-Dawley rats. The rats were then quickly euthanized and blood was collected. Compared to controls, the N₂O group had a three-fold increase in plasma CORT levels.

The present study sought to determine the effect of 60% N₂O on plasma CORT, EPI, and NE in rats by collecting blood samples prior to, and at three time-points during, a two-hour N₂O administration. Because these hormones are sensitive to the blood collection method as well as to other stressors such as restraint and handling (Vahl et al., 2005), the stress of repeated sampling was minimized by collecting samples remotely via flexible tubing connected to a previously placed indwelling jugular vein catheter, allowing rats to move freely without handling during the procedure.

Methods

Subjects

Male Long–Evans rats (n=9) arrived from Charles River Laboratories weighing approximately 300 g and were housed at 21 ± 1 °C on a 12:12 h light/dark cycle (lights on 0700–1900 h) with ad-libitum access to food and water in an AAALAC-accredited animal facility. All procedures were conducted in accordance with an approved protocol from the University of Washington's Institutional Animal Care and Use Committee.

Surgical Procedures

While under isoflurane anesthesia, each rat had a single surgery to place an indwelling jugular vein catheter using routine and previously described methods (Al-Noori et al., 2008).

Experimental Procedures

Following recovery from surgery, rats underwent a habituation procedure in which each rat was placed individually in a clear polycarbonate gas-tight testing chamber (Columbus Instruments, Columbus, OH, internal dimensions $18 \times 18 \times 31$ cm, volume of ~10 liters) with a lid that accommodated an infusion/withdrawal swivel (Instech Laboratories, Plymouth Meeting, PA, Model 375/20) for blood sampling. Habituation sessions, during which rats experienced the same procedures that would take place on the testing day (e.g., handling, familiarization with connection of the blood withdrawal lines, and flushing of catheters to mimic the blood sampling procedure), were performed daily for the 2–3 days before the N₂O session.

The N₂O test session (7 days post-surgery) began at 0900 h with a 2-h baseline period of control gas (21% oxygen, 79% nitrogen) followed by 2 h (1100–1300 h) of N₂O (60% N₂O, 21% oxygen, 19% nitrogen). The gas flow rate to the chamber was 1.6 liters per minute. N₂O and oxygen concentrations were measured from the exfluent gas line using an infrared gas analyzer (Normcapoxy, Datex, Helsinki, Finland). Blood samples (1.5 ml per sample) were collected at time t₀ (baseline, <10 min prior to N₂O delivery) and at t₃₀, t₆₀, and t₁₂₀ min during N₂O delivery. An equal volume of saline replacement was administered intravenously after each of the first three withdrawals to prevent hypovolemia associated with blood collection. Blood sampling and saline replacement were performed remotely via the flexible tubing connected to the jugular vein catheter. Blood for the catecholamine assays (i.e., NE, EPI) was collected in tubes containing EGTA-glutathione (Sigma). CORT samples were collected in tubes containing EDTA plus aprotinin (Sigma). Blood samples were centrifuged and plasma samples were stored at –80°C until assayed for circulating NE, EPI, and CORT levels. Assays for the first 5 rats were done using a radioenzymatic assay for determination of plasma EPI and NE, and a radioimmunoassay procedure for plasma CORT measurement, respectively. The assays used to analyze the blood samples from these initial 5 rats were no longer available when the blood samples collected from subsequently tested rats were ready for analysis. Therefore, the assays for the next 4 rats were performed using commercially available ELISA kits (BA E-5100 for EPI and BA-E-5200 for NE, Rocky Mountain Diagnostics, Inc., Colorado Springs, CO; EIA Kit K014-H1 for CORT, Arbor

Assays, Ann Arbor, MI). The values obtained using these different assay platforms were comparable (Table 1) and the data were consequently combined for analysis.

Data Analysis

Omnibus tests for the significance of the within-subjects hormone data were conducted with the linear mixed model in SPSS (version 24, IBM Corp., Somers, NY) with comparisons of individual time-points to baseline using LSD confidence interval adjustment. Significance was established at $p < 0.05$, two tailed. Data are reported as mean $\pm$ SE unless otherwise indicated.

Results

Plasma CORT, EPI, and NE increased significantly above baseline levels during the two-hour administration of 60% N₂O (Fig. 1). Plasma EPI returned toward baseline after the 30-min time-point whereas CORT and NE exhibited more sustained increases. Specifically, plasma CORT (n=9) was significantly elevated above baseline levels at all three time-points during N₂O inhalation (mixed model analysis, $p = 0.001$). Plasma NE (n=8) was elevated throughout the 120 min N₂O exposure with the values being significantly greater than baseline at 30 and 120 min ($p < 0.001$). Plasma EPI (n=8) was significantly elevated above baseline at the 30-min time-point ($p = 0.001$) but did not differ from baseline at the 60-min and 120-min time-points ($p > 0.05$). [The EPI and NE data from a single rat were excluded from the dataset because the assay results were extreme statistical outliers (i.e., the baseline EPI and NE values for this rat were > 5 standard deviations above the mean baseline value of the remaining 8 rats).]

Discussion

Administration of 60% N₂O to N₂O-naïve male Long-Evans rats significantly increases circulating levels of CORT, EPI, and NE. Both CORT and NE levels remained elevated throughout the two-hour N₂O administration. In contrast, although EPI levels were initially increased, they returned toward baseline values during the continued drug administration. Strengths of the current study include repeated blood sampling over time, lack of co-administration of other drugs with N₂O, and minimization of other potentially co-occurring stressful procedures or pharmacological effects that could impact hormone levels. A limitation of our study is the lack of a control group to demonstrate that the significant changes from baseline observed during N₂O inhalation are not due to factors such as the passage of time in the testing apparatus or the repeated blood sampling method and resulting decrease in erythrocytes.

The current finding that plasma CORT increases during 60% N₂O inhalation is congruent with a previous report in which rats were administered 80% N₂O (Fisher et al., 1987), and with the finding of Brandt et al. (1988) that N₂O caused a marked increase in plasma cortisol in conscious human subjects. However, our finding that NE and EPI levels increase during 60% N₂O does not fit with Baughman et al. (1990), in which 70% N₂O was administered to male Sprague-Dawley rats for 60 minutes and plasma EPI and NE were measured at 15 and 60 minutes during the N₂O exposure. Compared to controls, N₂O had no effect on EPI or

NE plasma levels at either time point (Baughman et al., 1990). However, the rats began testing after two hours of recovery from isoflurane anesthesia and surgical placement of catheters (left ventricle via the right carotid artery, both femoral arteries) and insertion of a rectal thermistor taped to the tail. Thus, the stress associated with recent anesthesia and surgery combined with the presence a rectal probe throughout the N₂O administration may have influenced the results.

Our interest in the stress-related neuroendocrine effects of N₂O is linked to N₂O's role as a drug of abuse and the potentially pathogenic role that stress systems are believed to play in the etiology of drug addiction. Koob et al. (2014) provide a recent view of stress within an allostatic model of addiction; '... drug addiction represents an excessive and prolonged engagement of homeostatic brain regulatory mechanisms This cascade of overactivation of the stress axis represents more than simply a transient homeostatic dysregulation; it also represents a dynamic homeostatic dysregulation that has been termed *allostasis*' (p. 379). Dysregulation is considered an important attribute of addiction (Koob & Le Moal, 2001; Lemieux & al Absi, 2016; Picetti et al., 2013) and a common feature of allostasis (Ramsay & Woods, 2014). Allostasis (Sterling & Ayer, 1988) was originally used in the context of the effects of stress on regulation, and contemporary views emphasize the importance of central stress effects and peripheral stress hormones as mediators of allostasis (Ganzel et al., 2010; McEwen & Wingfield, 2010). Glucocorticoids have been specifically implicated as an important factor influencing addictive vulnerability (Blaine & Sinha, 2017; Piazza & Deroche-Gamonet, 2013; Stephens & Wand, 2012; Picetti et al., 2013). For example, Koob et al. (2014) have hypothesized that, 'activation of the HPA response may be an early dysregulation associated with excessive drug taking' (p. 376). Understanding individual differences in addictive vulnerability is likely to be informed by research on individual differences in stress, a topic hypothesized to receive increasing research attention (Armario, 2015). Importantly, preclinical research models have an important role to play in understanding individual differences relevant to psychopathology (Armario & Nadal, 2013).

Research involving repeated administrations of N₂O to rats provides compelling evidence of drug-induced allostasis development. Dysregulatory overcorrections (sign-reversals) develop while inhaling 60% N₂O despite the maintenance of stable N₂O concentrations, implying that the observed sign-reversal is not a withdrawal phenomenon (Kaiyala et al., 2014; Kaiyala et al., 2012; Ramsay et al., 2005). The dysregulated inefficient concurrent activation of opposing behavioral and autonomic effectors during N₂O inhalation gradually develops over repeated N₂O administrations (Ramsay et al., 2014a). Furthermore, the effect of the motivated behavior offsets the dysregulated overactive autonomic effector activity that is responsible for the sign-reversal thereby providing evidence of the motivational significance of allostasis (Ramsay et al., 2014b). Emphasizing the relevance of drug-induced dysregulation to addiction, individual differences that predict allostasis development to N₂O have also been shown to predict individual differences in N₂O self-administration (Ramsay et al., 2015). It is an open question whether stress hormone changes during N₂O administration are involved in the development of N₂O-induced allostasis / dysregulation as suggested by the allostatic model of addiction. Considerable evidence supports the view that prolonged or repeated intermittent exposure to stressors can lead to maladaptive dysregulated physiological processes that result in a wide-range of pathologies (Herman,

2013). Based on the present findings that N₂O increases CORT, NE, and EPI, it is reasonable to consider N₂O as a physiological stressor.

The finding that an acute administration of 60% N₂O increases CORT, EPI, and NE provides a strong rationale for investigating whether these hormone levels change over repeated N₂O administrations. Future research should identify whether changes in any of these hormone levels are associated with the development of N₂O-induced allostasis and/or predict individual differences in N₂O self-administration.

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Fig. 1.

Plasma corticosterone (CORT; $n=9$), plasma epinephrine (EPI; $n=8$) and norepinephrine (NE; $n=8$) at baseline ($t=0$ min) and during initial 60% N_2O inhalation ($t=30, 60$ and 120 min) in rats. Values are mean $\pm$ SE and significance was determined by mixed model analysis. Time-points that differ significantly from baseline are indicated above bars. Note that EPI levels resolve more rapidly whereas CORT and NE exhibit more sustained increases.

Table 1.

Descriptive data according to type of hormone assay: Radioimmunoassay (RIA) for corticosterone (CORT); a Radioenzymatic Assay (RA) for epinephrine (EPI) and norepinephrine (NE); or commercial ELISA kits for CORT, EPI and NE. Blood samples were taken immediately prior to (baseline, t-0) and three times (t-30, t-60, and t-120 min) during a two-hour administration of 60% N₂O.

		Blood Sampling Time-Points (min)							
		t-0		t-30		t-60		t-120	
		RIA/RA	ELISA	RIA/RA	ELISA	RIA/RA	ELISA	RIA/RA	ELISA
CORT									
Blood samples (n)	5	4	4	4	4	4	4	4	4
Mean (ng/ml)	38.0	80.9	514.9	432.3	613.6	431.2	455.1	324.5	324.5
SD (ng/ml)	63.4	66.5	561.0	72.6	516.0	146.8	238.9	228.9	228.9
Median (ng/ml)	13.6	74.1	310.6	425.3	522.5	379.8	406.1	276.0	276.0
Range (min/max)	2.8/150.9	15.1/160.4	121.0/1317.4	351.2/527.4	91.8/1317.4	325.5/639.7	221.7/786.7	111.0/635.0	111.0/635.0
EPI									
Blood samples (n)	5	3	5	3	4	3	3	3	3
Mean (pg/ml)	170.4	52.5	430.2	362.1	314.6	144.2	202.3	122.8	122.8
SD (pg/ml)	126.5	25.9	291.2	160.9	332.5	71.4	148.9	102.3	102.3
Median (pg/ml)	114.0	59.7	264.0	321.5	185.2	130.6	147.0	149.6	149.6
Range (min/max)	34.3/334.0	23.8/74.2	162.0/760.9	225.4/539.4	91.1/797.0	80.5/221.4	89.0/371.0	9.7/209.0	9.7/209.0
NE									
Blood samples (n)	5	3	5	3	4	3	3	3	3
Mean (pg/ml)	236.8	239.8	374.8	548.8	350.9	328.7	434.4	433.1	433.1
SD (pg/ml)	85.7	134.9	130.7	23.8	81.9	116.1	159.2	27.6	27.6
Median (pg/ml)	206.4	226.8	352.9	537.0	340.8	289.0	481.2	420.4	420.4
Range (min/max)	177.0/388.3	111.9/380.7	227.0/519.1	533.2/576.2	264.0/458.0	237.7/459.4	257.0/565.0	414.1/464.7	414.1/464.7