A Systematic Review of Nitric Oxide’s Effect on Anxiety

Ali Azargoonjahromi¹

¹Affiliation not available

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Ali Azargoonjahromi*

* Shiraz University of Medical Sciences, Shiraz, Iran.

E-mail: aazargoonj@gmail.com

ORCID: 0000-0002-6997-9419

Abstract

Background

The incidence of anxiety disorders is rising on a global scale. On the potential function of nitric oxide (NO) in anxiety-like behavior, there is a notable amount of animal experimentation evidence with conflicting findings. The majority of this research examined the effects of NO, nitric oxide synthase (NOS) inhibitors, and NO donors on anxiety as well as their functions in various stress axis components. Notably, few studies have been conducted in humans to investigate the role of NO in anxiety. These studies are limited to examining plasma saliva and NO levels during an anxiety disorder as well as influencing the NOS1 gene on anxiety. Based on these studies, there is not enough evidence to reach a prevailing notion that NO is either anxiolytic or anxiogenic, or that NO can be conditionally anxiolytic or anxiogenic; thus, the available evidence needs to be analyzed systematically to reach a convergence conclusion.

Aim

The aim is to systematically review any potential link between NO and anxiety in both humans and animals.

Method

Seven human and thirty-seven animal original studies have been included based on specific selection criteria. Among the original human studies selected for analysis, three were cross-sectional studies, two were case control studies, and two were cohort studies. The Google Scholar and PubMed databases were searched for studies that have been written in English and published in peer-reviewed journals with no restrictions on the date of publication. Additionally, this project has been reported in accordance with PRISMA guidelines and PICOS criteria.

Results

The level of NO is associated with the pathophysiology of anxiety. Although this association seems plausible, the results of analyses in human studies, due to their limitations, by and large do not provide strong evidence for the correlation of NO with anxiety. However, most animal studies reached the conclusion that NO induces anxiety-like behavior.
**Conclusion**

The results provide plausible, albeit not enough, evidence for the correlation of NO with anxiety.

**Keyword**: Anxiety; Nitric Oxide; Anxiety-like Behavior; Nitric Oxide Synthase Inhibitors; Nitric Oxide Donor

1. Introduction

1.1. Nitric Oxide

The gaseous signaling molecule nitric oxide (NO) is created by the enzyme nitric oxide synthase (NOS), which is found in neurons and perivascular tissues (1). There are three primary isoforms of this enzyme: neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (2). NO is recognized to be both an atypical neurotransmitter and a reactive oxygen species (ROS) in both the peripheral nervous system (PNS) and central nervous system (CNS), and it has a significant physiological role to play in the cardiovascular system (3-5). Both enzymatic and non-enzymatic methods are used to control endogenous NO production. Through a number of redox reactions, such as the conversion of L-arginine into L-citrulline and NO in the presence of oxygen and NADPH, NOS catalyzes the enzymatic synthesis of NO (6, 7). NOS1 and NOS3 are constitutive Ca2+/calmodulin-dependent enzymes, whereas NOS2 is Ca2+-independent. The N- and C-terminal oxygenase and reductase domains are present in each subunit of the homodimeric enzyme. The former has binding motifs for the cofactor tetrahydrobiopterin (BH4), the substrate L-arginine, and heme (8).

Nitrates (NOx), the end metabolite of NO, can be used to assess the amount of NO in peripheral and central tissues (9, 10). NOx can also be found in saliva since salivary glands actively take circulating nitrates and release them into saliva (11). nNOS isoforms are distributed differently in various parts of the brain and spinal cord. Recent studies have revealed that impulsivity and related psychopathology are affected by a dinucleotide variable number tandem repeat (VNTR) polymorphism in the promoter region of the alternative first exon 1f (ex1f) of the human neuronal nitric oxide synthase (NOS1 ex1f-VNTR) (12, 13). As a proxy for NO production in humans, the genotype of the functional promotor polymorphism NOS1 ex1f-VNTR is identified, particularly in specific brain regions such as the cortex, striatum, and hippocampus (14-16). It is worth noting that NO indirectly triggers the nucleus accumbens’ release of acetylcholine by stimulating adjacent glutaminergic neurons (17).

1.2. Anxiety

Anxiety (disorders) – deemed the most prevalent mental health condition (18) – are a cluster of mental disorders characterized by uncontrollable and remarkable feelings of anxiety and fear that affect the quality of life (19). The disease known as a panic attack or panic disorder (PD) is characterized by recurrent episodes of severe anxiety, fear, or terror that peak within minutes (20). Notably, fear and anxiety are distinct emotions because fear is the emotional response to a present threat, whereas anxiety is the anticipation of a potential threat (21). Anxiety is accompanied by autonomic symptoms like palpitations, sweating, headaches, slight stomach discomfort, and tightness in the chest (22). Anxiety can also be categorized as “trait” anxiety and “state” anxiety, which refer to a fairly constant personality trait and a brief outburst of insecurity, respectively (23). Indeed, when this emotion is abnormal, overpowering, continuous, and out of proportion to threat, it is classified as having an anxiety condition (disorder or disease) (24, 25).

Anxiety involves brain structures that regulate the hypothalamic–pituitary–adrenal (HPA) axis, viz., limbic structures such as the hypothalamus, hippocampus, amygdala, and periaqueductal gray (26) to regulate the
stress- and fear-related anxiety responses through their interconnections with higher cortical areas and the lower brain stem.

Along with emotional factors, environmental factors affect the functioning of the limbic system and its connections, increasing the risk of anxiety disorders (27, 28). In addition, the initiation of fear responses may be regulated by complex neurotransmitter systems. For example, amino acids (glutamate and GABA), biogenic amines (histamine-catecholamine and serotonin), and neuropeptides can interact at different levels in the brain to influence anxiety-related responses (29, 30). Of note, the underlying causes and mechanisms are relatively unknown, even though various clinical and preclinical studies related to anxiety disorders have been conducted.

1.3. NO and Anxiety – Animal Studies; Conflicting Findings; Human Studies

The brain structures such as limbic areas, with their connections, are encompassed high amount of NOS enzyme (31).

On the potential function of NO in anxiety-like behavior, there is a notable amount of animal experimentation evidence with conflicting findings. The majority of this research examined the effects of NO, NOS inhibitors, and NO donors on anxiety as well as their functions in various stress axis components. Studies are currently being conducted to define NO’s role in this psychopathology (32) since some have shown anxiolytic effects of NO (32-35), while others have suggested anxiogenic effects of NO (36-38).

For instance, the NOS inhibitor L-NAME has been demonstrated to reduce adrenocorticotropic hormone (ACTH), also known as a hormone controlling the production of cortisol and a so-called stress hormone, in response to shock and to lessen upregulation of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) gene expression in the hypothalamic paraventricular nucleus after exposure to neurogenic stressors; NO has a stimulating effect on the hypothalamus in reaction to stress and anxiety, which is why it was shown that giving rats NO intracerebroventricularly increased the quantity of CRH and AVP transcripts in the paraventricular nucleus (39), whereby NO has a stimulatory role in the hypothalamus in response to stress and anxiety. In contrast, in another animal investigation, L-NAME enhanced pituitary ACTH production in response to AVP and circulating proinflammatory cytokines, indicating that NO plays an inhibitory effect at the central or pituitary level (40). Another illustration from animal studies shows that low levels of NO have been demonstrated to decrease GABA release in a Ca2+- and Na+-dependent way, whilst high levels of NO have been shown to promote GABA release (41).

Few studies have been conducted in humans to investigate the role of NO in anxiety. These studies are limited to examining plasma saliva and NO levels during an anxiety disorder as well as influencing the NOS1 gene on anxiety. The neuronal nitric oxide synthase (NOS1) gene has been suggested as a potential hereditary risk factor for anxiety disorders. The NOS1 gene was discovered to encode the neuronal isoform of nitric oxide synthase (NOS-I), which is widely expressed throughout the human brain, particularly in the frontal cortex, basal ganglia, cerebellum, and hippocampus (42), despite the lack of biomarkers or safe drugs that target NOS-I (15). NOS-I catalyzes the production of the neurotransmitter NO (42).

Based on these studies, although there is not enough evidence to support the notion that NO is either anxiolytic or anxiogenic, most of them resulted in anxiety-like behavior in the presence of NO (43-47). On the basis of the equivocal nature of the data available from in vivo studies, the current view proposes that NO has differential effects on the different components of the stress axis (48). To reach a convergence notion, further studies are in need to be carried out in humans.

1.4. The Current Study

An extensive systematic review of the relevant literature is one way to give a more conclusive response to the question of whether NO and anxiety disorders are related. These analyses were set out to provide information on both human and animal studies, in separate subheadings, on the relationships between the following variables: In human studies: (a) anxious situations and plasma nitrate levels (NO indicator); (b) the effect of NOS1 ex1f-VNTR on anxiety; and (c) anxious situations and salivary NO levels. In animal
studies: (a) the effect of NOS inhibitors and nitric oxide donors on anxiety situations; (b) the trend of NOS gene expression during facing anxiety situations; (c) NOx levels during facing anxiety situations; (d) protein c-FOS level as a result of facing anxiety situations.

2. Methods and Materials

This project was reported following the guidelines of PRISMA (49) and PICOS criteria. PRISMA checklist can be found in Appendix 1. PICOS criteria can be shown in tables.

2.1. Search Strategy

The Google Scholar and PubMed databases were searched—without any limit regarding date of publication—for eligible papers using the following terms: (Nitric oxide), (nitric oxide syntheses), (nitric oxide donor), (nitric oxide inhibitors), (NOS1 gene), and (NOS1 exIf-VNTR) hedged in by (anxiety) and (anxiety-like behavior) and (anxiety disorder) and (panic disorder). The reference lists of identified articles were scrutinized, obtained additional published studies by reviews for references.

2.2. Selection Criteria

Original results that looked into the connection between nitric oxide and anxiety in both animals and humans were incorporated. For inclusion, studies must be written in English, published in peer-reviewed journals, including early online publication. Review articles, letters, comments, abstracts, and those articles in which anxiety was not one of their outcome factors were also disregarded.

A total of 71 studies were obtained through the Google Scholar and PubMed databases, and a total of 4 additional articles were identified through scrutinizing the reference lists of the identified articles. Out of all these 75 articles, 11 were original human studies, 53 were original animal studies, and 11 were review, letter, or comment articles.

Out of 11 human articles, the complete texts of these 11 articles were carefully read, out of which two were excluded because they used an extract as exposure that contained many other elements in addition to nitric oxide donor, making it difficult to determine whether the observed effects were caused by nitric oxide donor or by the other components. Further, two of them were disqualified because they provided insufficient data. Thus, a total of 7 human studies were evaluated in this article.

Out of 53 animal studies, ten were excluded since they used other components besides NOS inhibitors, NO donors, or NOx, making it difficult to determine whether the observed effects were caused by which of these components. Besides, six of these studies were excluded because they did not provide sufficient data. Hence, a total of 37 animal studies were evaluated. Figure 1 shows the flowchart indicating how pertinent studies were selected.

**Figure 1.** Flowchart of the identification and selection of relevant studies.
2.2.1 Human Studies

The following new findings in human studies were also presented: (a) the relationship between anxious circumstances and salivary NO levels; (b) the effect of NOS1 ex1f-VNTR on anxiety; and (c) the relationship between anxious circumstances and plasma levels of nitrate (NO indicator).

The selected papers used sundry methods to measure the indication of NO level, as follows: Plasma nitrite (as indicators of NO production) levels which were measured with Griess reaction (44); salivary nitrate levels were determined with a colorimetric assay with a Griess reagent (43, 50, 51); and genotyping performed on blood samples determining NOS1 ex1f-VNTR by PCR amplification (45-47).

To measure symptoms and severity of anxiety in human studies, they used DSM-IV criteria (44), the Competitive State Anxiety Inventory-2 (CSAI-2) (51), the Arabic version of the Perceived Stress Scale (PSS) (43), DSM-IV-TR criteria (American Psychiatric Association 2000) and the Composite International Diagnostic Interview (CIDI) (47), The Spielberger State Anxiety Inventory (SSAI) was used at ages 15 from 18 (46), and the Spielberger Trait Anxiety Inventory (STAI) at age 18 (46), and the Arabic version of the Hamilton Anxiety Rating Scale (HAM-A) (50).

2.2.2 Animal Studies

The following new findings in animal studies were also presented: (a) the effect of NOS inhibitors and nitric oxide donors on anxiety situations; (b) the trend of NOS gene expression during facing anxiety situations; (c) NOx levels during facing anxiety situations; and (d) protein c-FOS level as a result of facing anxiety situations.

The selected papers used sundry methods to measure the indication of NO effects on anxiety, as follows: The role of NOS inhibitors or NO donors in the behavior of animals when they are faced with anxiety situations (32, 33, 36-38, 52-78); two of which evaluated the level of NO or NOx as a result of confronting anxiety circumstances (79, 80); two of which assessed the trends of NOS gene expression when animals are faced with anxiety situations (81, 82); protein c-FOS level as a result of facing anxiety situations (83).

To measure symptoms and severity of anxiety in these animal studies, the studies used Elevated Plus Maze (EPM) (36-38, 53-63, 65-80, 82, 83), plasma cortisol measurement (60), Light-Dark Test (LDT) (32, 33, 37, 52, 56, 58, 64, 70, 76), Open Field Test (32, 33, 37, 38, 52, 55, 61, 64, 68-72, 82), the small platform (SP)
technique as a stress model (65), Running Test (81), Freezing Behavior Test (53), Social Interaction Time (37, 70, 73), Novelty Suppressed Feeding (NSF) (82), Predator Exposure Box (79), and Vogel Punished Licking Test (55).

3. Results

3.1. Human Studies

Among the seven final epidemiological articles selected for analysis, three of which were cross-sectional studies (43, 50, 51), two were case control studies (44, 47), and two were human cohort studies (45, 46). One of these studies measured the plasma levels of NO (plasma nitrite as indicators of NO production levels) in individuals in anxious state (44), three assessed the effect of NOS1 ex1f-VNTR on anxiety (45-47), and three of studies investigated the salivary levels of NO in anxious state (43, 50, 51).

3.1.1. Anxious Situations and Plasma Levels of Nitrate (NO Indicator)

Little is known about the total nitrate level, which is an indicator of NO, in patients with anxiety. One case-control study carried out in adults found that the NO levels of the patients were higher than those of the control subjects, even though the difference was not statistically significant (44). In addition, after eight weeks of antidepressant treatment, a noticeable decline in the level of NO was reported in patients with panic disorders (44). Methodological details and results of the analyzed study thereof has been summarized in table 1, followed PICOS criteria.

<table>
<thead>
<tr>
<th>Author, (year)</th>
<th>Patients</th>
<th>Objective</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herken et al. (2006)</td>
<td>32 patients (18 females, 14 males; age range, 16 – 54 years) diagnosed with PD according to DSM-IV criteria</td>
<td>NO levels of the patients were higher than those of the control subjects; also, this level decreased significantly after antidepressant treatment.</td>
<td>(44)</td>
</tr>
</tbody>
</table>

3.1.2. The Impact of NOS1 ex1f-VNTR on Anxiety

As to three studies carried out in human – one cross-sectional, one case control, and one human cohort, NOS1 ex1f-VNTR allelic variation differentially impacts on the hippocampus and amygdala during anxious apprehension and fear conditioning (45-47). In these human studies, it was discovered that in healthy people, the short (S) allele of a functional promotor polymorphism of NOS1 (NOS1 ex1f-VNTR) was linked to increased anxiety and altered fear conditioning in the amygdala and hippocampus.

According to a large human cohort carried out in 1019 individuals (45), the NOS1 ex1f-VNTR S-allele has a recessive impact on apprehensive apprehension as demonstrated by the NOS1 ex1f-remarkable VNTR’s impact on trait anxiety and considerable contributions from trait anxiety. Moreover, it was discovered in this cohort study that short allele carriers exhibited higher levels of neuroticism and anxiety than participants with the long/long (l/l) genotype; S-allele carriers demonstrated substantially higher subjective anxiety ratings. In essence, this study demonstrated that NO signaling affects nervous apprehension in a noticeable way.

In the other human cohort study that evaluated the effect of the NOS1 ex1f-VNTR genotype on anxiety (46), females in this study scored higher on state anxiety than males, and this anxiety score also diminished with age, demonstrating an astonishing main effect of the NOS1 genotype on state anxiety.

In a case control study, patients (n = 48) and healthy control subjects (n = 34) with differentiation only in education, that investigated how NOS1 ex1f-VNTR genotypes are associated with differential neural activation in amygdala and hippocampus during fear condition; they discovered that individuals with PD and agoraphobia experience distinct effects of NOS1 ex1f-VNTR allelic variation on the amygdala and hippocampus during the conditioning and extinction of fear than do healthy control participants. Of note, following cognitive behavioral therapy (CBT), patients’ genotype-associated effects remained undeveloped.
Methodological details of these studies mentioned in this section has been summarized in table 2, followed PICOS criteria.

**Table 2.** Methodological details of studies investigated NOS1 ex1f-VNTR genotypes association with anxiety.
<table>
<thead>
<tr>
<th>Author, (year)</th>
<th>Participants/patients</th>
<th>Controls</th>
<th>Objective</th>
<th>Duration</th>
<th>Limitations</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuhn et al. (2016)</td>
<td>1019 participants</td>
<td>/</td>
<td>NOS1 ex1f-VNTR genotypes association with anxiety</td>
<td>Not mentioned</td>
<td>Although exploratory analyses have been included to investigate a transdiagnostic impact of this genetic variant, despite being highly correlated with anxiety, our analyses were restricted to the affective domain only. The NOS1 ex1f-VNTR S-allele was found to have a dominant effect on behavioral studies of experimental context conditioning, despite having a recessive effect on anxiety- and depression-related traits.</td>
<td>A robust impact of NOS1 ex1f VNTR on anxious apprehension.</td>
<td>(45)</td>
</tr>
<tr>
<td>Author, (year)</td>
<td>Participants/patients</td>
<td>Controls</td>
<td>Objective</td>
<td>Duration</td>
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<tr>
<td>Kurrikoff T et al. (2012)</td>
<td>593 participants</td>
<td>/</td>
<td>NOS1 ex1f-VNTR genotypes association with anxiety</td>
<td>From 2004 to 2007</td>
<td>Not mentioned</td>
<td>Finding a significant main effect of the NOS1 genotype on state anxiety; this effect (effect of being a short carrier) was unrelated to neuroticism and both adaptive and mal-adaptive impulsivity.</td>
<td>(46)</td>
</tr>
</tbody>
</table>
### 3.1.3. Anxious Situations and Salivary Levels of NO

Few studies have been carried out to investigate if salivary nitrate correlates to anxiety in a group of human subjects. According to two cross-sectional studies that investigated the levels of salivary nitrates (an indicator of NO) in patients with acute anxiety (43, 51), a significant increase in the salivary NOS activity and NO level was shown; these increased levels have been related to increased anxiety. Of note, in one of these studies (51), a noticeable decrease in arginase activity was shown; this decreased arginase activity seems to be a major reason for increased NO production.

Although all previous studies focused on salivary nitrate levels (an indicator of NO) in acute anxiety models, a cross-sectional study (50) was the first human study to investigate the correlation between salivary nitrates and daily psychological anxiety, not acute or pathophysiological anxiety. This study, which examined 73 participants, showed that there is no significant correlation between salivary nitrates and daily psychological anxiety. Methodological details of these three cross-sectional studies have been summarized in Table 3, followed PICOS criteria.

Table 3. : Methodological details of studies investigated salivary levels of NO correlation with anxiety.
<table>
<thead>
<tr>
<th>Author, (year)</th>
<th>Participants/patients</th>
<th>Objective</th>
<th>Limitations</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gammoh OS et al. (2015)</td>
<td>70 participants (40 of whom was male, 30 female)</td>
<td>Assessing oxidative stress biomarker (salivary NOx as an indicator of NO) in a group of refugees with acute anxiety</td>
<td>Disregarding additional anxiety-related aspects including antioxidant indicators (catalase, super oxide dismutase, and their relationships with other factors like sleep difficulties or underlying medical concerns).</td>
<td>Participants who experienced anxiety showed a significant increase in salivary nitric oxide; NO may be a potential biomarker for anxiety disorder.</td>
<td>(43)</td>
</tr>
<tr>
<td>Ulkar B et al. (2012)</td>
<td>18 participants aged 18 to 29 years</td>
<td>Investigating salivary NO metabolism and its association with the periodontal reaction to anxious situation</td>
<td>Not mentioned</td>
<td>It has been demonstrated that higher levels of anxiety are correlated with a considerable rise in the salivary NOS activity and NO level as well as a decrease in arginase activity.</td>
<td>(51)</td>
</tr>
<tr>
<td>Gammoh OS et al. (2016)</td>
<td>73 participants</td>
<td>Determining whether salivary nitrate in a sample of human individuals connects with their everyday psychological stress and distress</td>
<td>Not mentioned</td>
<td>No significant correlation exists between salivary nitrate and daily psychological stress and anxiety.</td>
<td>(50)</td>
</tr>
</tbody>
</table>

3.2. Animal studies
Among the thirty seven final articles selected for analysis, thirty two of them assessed the role of NOS inhibitors or NO donors in the behavior of animal when they are faced with anxiety situations (32, 33, 36-38, 52-78), two of which evaluated the level of NO or NOx as a result of confronting anxiety circumstances (79, 80), two of which assessed the trends of NOS gene expression when animals are faced with anxiety situations (81, 82), and also one evaluated the level of protein c-FOS as a result of facing anxiety situations (83). The summarized results are as follows:

### 3.2.1. The role of NOS inhibitors or/and NO donor in the behavior of animal faced with anxiety situations

A total number of thirty-two animal studies were assessed in this category (32, 33, 36-38, 52-78). According to the results of such studies, it was found that the role of NOS inhibitors and NO donors in anxiety-like behavior is a contradictory subject. This means that NOS inhibitors and NO donors have shown sundry effects on the behavior of animals in studies—anxiolytic effects in some studies and anxiogenic effects in some other studies.

#### 3.2.2. The role of NOS inhibitors in anxiety

Thirteen studies (36, 37, 55-58, 60, 69, 70, 72, 75, 78) resulted in anxiolytic effects of NOS inhibitors. To explain more, it was revealed that when they used NOS inhibitors like L-NAME, 7-NI, Carboxy-PTIO, TRIM, aminoguanidine, or L-NOARG, the time animals spent on open arms in EPM test increased. In addition, administering such drugs led to increasing the time spent in light zone in Light-Dark Test. Along with these studies, three studies (36, 58, 70) examined the levels of NOx after injection of NOS inhibitors and found that NOS inhibitors like aminoguanidine and L-NAME can attenuate the stress-induced anxiety-like behavior and decrease plasma nitrite levels.

However, six studies (54, 63, 67, 73, 76, 77) end to the result that NOS inhibitors like L-NAME or L-NOARG injection can decrease the time when animals spend on open arms in EPM test, or decrease the time animal spent on light zone in Light-Dark Test; thus, the reported the anxiogenic effect of NOS inhibitors.

Of note, five (59, 62, 68, 71, 74) of all these studies came into the result that NOS inhibitors either do not have any impact on anxiety-like behavior of animals or affect anxiety-like behavior in dose-dependent. For instance, in one of these studies, it was reported that the NO synthase inhibitors, per se, do not have any significant effect on the on the EPM parameters as compared with control values. The data for open arm entries and open time were 18.6 ± 5.2 and 9.0 ± 2.4, respectively, for L-NAME and 16.8 ± 5.8 and 7.5 ± 1.8, respectively, for 7-NI (p > 0.05 in each case) (59). It also was found in another study that L-NOARG, an inhibitor of NO synthase, was administered at dosages ranging from 30 to 120 mg kg⁻¹. These levels reduced the percentage of entries and the amount of time spent in the open arms of the labyrinth, but all except 30 mg also reduced the number of entries into enclosed arms. When the rats were evaluated after receiving chronic L-NOARG (an inhibitor of NO synthase), these effects vanished (3.75 to 60 mg kg⁻¹ IP, twice a day for four days) (62). The other example is that in the EPM test, the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) (50 mg/kg) exacerbated the effects of restraint stress, but the lower dose (10 mg/kg) of the medication alleviated the same (71).

Of interesting, in a study (65), in control mice, the NOS inhibitors 7-NI (20.0-120.0 mg/kg) and L-NOARG (20.0 and 40.0 mg/kg) generated anxiolytic effects, but the NOS inhibitor L-NAME (20.0 and 40.0 mg/kg) induced anxiogenic effects. NOS inhibitory effects were altered in mice during SP stress. The dose of 7-NI that caused an anxiogenic effect in SP-stressed mice was 20.0 mg/kg, in contrast to control mice, and all other doses of 7-NI, with the exception of 80.0 mg/kg, as well as L-NOARG and L-NAME, had no impact.

### 3.2.3. The role of NO precursors or NO donors in anxiety

Out of thirty-two studies in this category, thirteen of which investigated the impact of NO donors or NO precursors injection on anxiety-like behavior of animals. Six studies (53, 57, 61, 63, 77, 78) end with the result that the injection of NO precursors/ donors like sodium nitroprusside (SNP), L-arginine, NOC-9, or sildenafil culminate in increased anxiety. It was due to animals spending less time in the light zone in the
Light-Dark Test and on open arms in the EPM test. Some studies reported that NO precursors/donors can decrease anxiety in animals. It was concluded because, by injecting them, the time when animals spent on open arms in the EPM test increased, and in one study (32) the time spent on the central zone in the open field test was shown to be significantly higher than that in the edge zone.

Three studies (33, 38, 66) reported that NO precursors/donors’ injection did not influence the first latency to enter the dark chamber and the number of transitions between the light and dark compartments of the apparatus in the light/dark test and did not modify the number of squares crossed, grooming episodes and rearing in the open field test or did not modify the behavior of animals in EPM test. For instance, intra-LmPFC injections of NOC-9 (an NO donor) (0, 37.5 or 75 nmol) did not change anxiety indices [%OE: F(2,28)= 1.12; p= 0.34; %OT: F(2,28)= 0.21; p=0.80] or general locomotor activity [CE: F(2,28)= 0.94; p=0.40] in mice exposed to the EPM (66).

Of note, it was resulted that the NO precursor L-arginine (200 mg/kg i.p.) does not affect the behavior of animals in the EPM test, but sildenafil (1 mg/kg i.p.) combined with L-arginine (200 mg/kg i.p.) dramatically reduced the amount of time spent in the open arms and the percentage of open arm visits (38).

In one study (52), however, results were dependent on dose: acute administration of 1 mg/kg (NO donor sodium nitroprusside) SNP 30 but not 60 min before testing induced anxiolytic-like behavior. In contrast, a single injection of 3 mg/kg SNP 30 minutes before testing decreased the activity of the rats overall, whereas at 60 minutes prior to testing, this dose had no effect on the animals’ performance in the light-dark or motor activity tests.

3.2.4. The level of NO or NOx as a result of confronting anxiety circumstances

One study (80) reported no significant change in NOx levels in the 359 vMPFC after anxiogenic-like behavior was evaluated 24 h 360 after RS in animals single-housed. In the other study (79), male Wistar rats were evaluated for high EPM seven days after being exposed to a toy or live cat for ten minutes. Their brains were taken out immediately after the test to be examined for neuronal NOS (nNOS) immunohistochemistry and nitrite/nitrate (NOx) levels. The presence of the live cat induced more freezing reactions. One week later, the animals that froze when they saw the cat showed a reduced number of entries in the EPM’s open arms and an increase in nNOS-positive neurons in the PFC and basolateral amygdala, but not in the hippocampus, central and medial nuclei of the amygdaloid complex, or dorsal-lateral periaqueductal grey. Furthermore, a week after being exposed to cats, animals displayed elevated NOx levels in the PFC but not in the hippocampus. When exposed to cats, the number of nNOS neurons and NOx levels in the PFC significantly correlated with the duration of the freezing phase.

3.2.5. The trends of NOS gene expression when animals are faced with anxiety situations

To evaluate the NOS gene expression trend when facing anxiety situations, two studies have been conducted. In one of which (81), reduced nNOS expression levels in the cerebellum but not in the cortex were reported as a result of Enriched Environment (EE), whereby EE can reduce anxiety-like behaviors in aged mice. Likewise, by assessing anxiety-related behaviors using novelty suppressed feeding, open-field, and EPM in the other study (82), mice lacking the nNOS gene displayed an anxiolytic-like phenotype, implicating nNOS in anxiety.

3.2.6. The level of protein c-FOS as a result of facing anxiety situations

Male Wistar rats were given a 15-minute exposure to the EPM, and two hours later their brains were removed and processed for c-Fos immunohistochemistry and NADPH-diaphorase histochemistry (a measure of neuronal functional activation) (NADPH-d; used to detect the presence of NOS neurons). In contrast to the amygdaloid complex, bed nucleus of stria terminalis, dorsal premammillary nucleus of the hypothalamus, and inferior colliculus, exposure to the EPM significantly increased double-stained cells (cFos + NADPH-d positive neurons) in the parvocellular paraventricular (pPVN) and lateral (LH), as well as the dlPAG and dorsal raphe nuclei. These findings imply that exposure to an EPM activates neurons that contain NOS in parts of the brain associated with fear and anxiety (83).
4. Discussion
This systematic review comprised seven investigations, including three cross-sectional, two case-control, and two human cohort studies that have been evaluated in the human studies category, while thirty-eight animal studies have been assessed in this systematic review. The results are summarized and discussed below.

4.1. Human Studies
Through a case-control study, the researchers discovered that patients with anxiety disorders (panic disorder) can have higher levels of total nitrite, which is a marker of NO, but not to a significant level (44). In contrast to the elevated NO levels in schizophrenia and bipolar affective disorder (BPAD) during manic episodes (84-87), unaltered NO levels in panic patients suggest that PD is not as destructive as schizophrenia and bipolar disorder. These events augment ROS interactions with NO function as well as the oxidative stress activity of NO (3, 4). Antidepressants were also found to diminish the level of NO in patients with PD (44), indicating the anti-oxidative function of antidepressants (88).

The findings are that NOS1 ex1f-VNTR allelic variation has different effects on the amygdala and hippocampus during anxious anticipation and fear training, which is consistent with our expectations (44-46). Additionally, persons with the short/short (s/s) genotype were seen to be more neurotic and anxious (46). The findings of the current research suggest that in the general population, the functional NOS1 ex1f-VNTR polymorphism is linked with state anxiety as well as basic personality traits and impulsivity. As a result, those who have the NOS1 short allele should be more susceptible to environmental stressors. Overall, the existence of two ex1f-VNTR short alleles was linked to known pathological anxiety sensitivity variables such trait anxiety and trait worrying.

As to the studies reviewed (three cross-sectional studies), it was found that salivary NO levels can be deemed a biomarker of anxiety. However, it is of note that the levels of salivary NO are dependent on scores of anxiety, as participants faced daily psychological anxiety and showed no significant correlation (50). In contrast, the amount of salivary NO in participants with severe anxiety was shown to be remarkable (43, 51). People with anxiety have increased salivary NOx concentrations, which indicates an oxidative stress state. DNA fragmentation, lipid peroxidation, and mitochondrial dysfunction are all caused by the conversion of too much NOx into peroxinitrite (ONOO-) (89, 90). One study (51), which found a drop in arginase, suggests that arginase activity may play a significant role in the rise in NO production. Arginase competes with NOS for arginine, depletes it, and therefore regulates NO production.

4.2. Animal Studies
According to studies reviewed, the inhibition of NO has different effects on anxiety-like behavior, even though most of the studies—thirteen/thirty-two—noted that NOS inhibitors have anxiolytic effect in animals. By injection of NOS inhibitors in sundry anxiety tests, animals showed different behaviors; this means that they showed decreased/increased/no change in anxiety-like behavior—not to mention that most studies (36, 37, 53, 55-58, 60, 69, 70, 72, 75, 78) reported the anxiolytic behavior of NOS inhibitors. And also, in some studies (59, 62, 68, 71, 74), the efficacy of NOS inhibitors was dose-dependent or not significant.

Turning to the impact of NO precursors/donors on anxiety, the studies had contradictory results, but most of them (53, 57, 61, 63, 77, 78) reported anxiogenic effects of NO precursors/donors.

Few studies have been carried out to investigate the level of NO or NOx as a result of anxiety situations in animals. According to the two studies (79, 80) that were evaluated, facing anxiety situations can increase the level of NOx in the brains of animals. The discovery was that facing anxiety situations can increase NOS gene expression, leading to elevated levels of NO, which have anxiogenic effects (81, 82). The protein c-FOS, which is a high-resolution marker of neural activity, can be shown during stress and anxiety situations (91, 92). Also, c-FOS is regulated by NO in nucleus tractus (93), so an increased level of it shows high activity of NO, thereby affecting anxiety-like behavior. c-FOS levels were found to be increased as a result of stressful situations. These findings imply that exposure to an EPM activates neurons that contain NOS in parts of the brain associated with fear and anxiety (83).
4.3. Probable mechanisms by which NO is related to anxiety

4.3.1. Microglial activation and release of proinflammatory cytokines

Stressful situations can initiate both direct and indirect activation of microglia, resulting in the secretion of harmful proinflammatory cytokines. The activation of neurons resulting from stress leads to regionally specific associations with variations in the morphology of microglia. The phenomenon of increased cell size, along with thickening and shortening of cell processes, is attributable to a process known as deramification of microglia, which facilitates direct activation of microglia (94). The activated microglia demonstrate an inclination towards heightened levels of inflammation and phagocytosis (95). Consistent with this observation, it has been discovered that enriched microglia obtained from the brains of stressed mice are correlated with elevated expression of genes associated with proinflammatory cytokines, as well as amplified expression of markers of inflammation (94, 96). In short, the activation of microglial cells and subsequent initiation of neuroinflammatory reactions may result in the occurrence of anxiety induced by stress. This phenomenon reflects the potential connection between microglial activity and the development of stress-related psychological conditions.

The glial cells are attributed with the encephalic localization of iNOS. The activation of microglia is closely linked to psychophysiological stress, and is brought about by the increased release of corticosterone, norepinephrine, and adrenaline (97). The subsequent release of proinflammatory cytokines after microglial activation causes glial or astrocytic iNOS to be directly activated, which facilitates increased NO production from L-arginine. Once activated, iNOS promotes significant NO generation for extended periods of time until substrate depletion (98). The expression and activity of iNOS are clearly upregulated in the cerebral cortex after a single 6-hour acute immobilization stress. The nuclear factor kappa light chain enhancer of activated B cells (NF-κB), a transcription factor, is innervated after the NMDA receptor, which in turn mediates the increase in iNOS expression (99). Through overexpression of TNF-convertase, acute stress-induced NMDA receptor activation also raises levels of tumor necrosis factor-alpha (TNFα). It has been shown that the stress-induced translocation of NF-κB and consequent iNOS expression can be carefully regulated by antagonistic TNF-convertase. This reaffirms the contribution of TNF to the increase in iNOS expression during stressful situations (100).

4.3.2. HPA axis activation

Various physiologic changes in the body have been linked to stress. Stress activates the HPA axis and sympathoadrenomedullary system (SAS), which releases catecholamines as well as the stress chemicals glucocorticoids and corticosterone from the adrenal gland (101). The hypothalamic paraventricular nucleus (PVN) is thought to be where the HPA stress response reaches its apogee. ACTH is released into the bloodstream by the anterior pituitary gland in response to the production of CRH from the parvocellular neurosecretory neurons. As a result, the release of corticosterone occurs when the adrenal gland becomes more sensitive to ACTH. The hippocampus and amygdala contain particular mineralocorticoid and glucocorticoid receptors (GRs) that are involved in the control of fear and anxiety-like behavior. These GRs are also implicated in the action of corticosteroids. As a result of the HPA axis activation and increased corticosterone production, specific brain regions that control anxiety and depression have been shown to undergo oxidative stress conditions. Oxidative free radicals accumulate as a result of oxidative stress. Additionally, it has been discovered that the activation of the HPA axis and increased corticosterone release causes oxidative stress conditions in particular brain regions responsible for anxiety and depression. Another mechanism of stress-induced anxiety may be the buildup of reactive free radicals that are produced as a result of oxidative stress, which causes neuronal damage (102).

NO dysfunctions may alter neurotransmitters and hormones to impact anxiety-like reactions. Though not exclusively, NO is abundant within the PVN and is closely connected to the HPA axis (39). The PVN is a significant source of CRH, which stimulates the pituitary’s release of ACTH, which in turn activates cortex’s release of cortisol (or corticosterone) (103). Even though cortisol has been strongly linked to the development of affective disorders, the central effects of CRH may be crucial in the development of anxiety and anxiety-
like behaviors (104). The central nucleus of the amygdala, which is a major player in the autonomic and behavioral responses to frightening stimuli, contains CRH terminals, cell bodies, and receptors (105). The CRH system is distributed throughout the brain (106, 107). Less emphasis has been paid to the alterations that actually occur in brain regions linked to fear and anxiety, despite the fact that numerous research has examined the effects of NO inhibition on anxiety-like reactions. Many of the research described above also do not take into account how social living conditions affect behavior.

4.3.3. Oxidative stress

Oxidative stress significantly contributes to neuronal degeneration in the central nervous system because encephalic areas have been identified to be sensitive to oxidative free radicals (108). Chronic stress substantially increases the relative production of ROS, which are characterized by elevated levels of nitrite and lipid peroxidation. Stress has been linked to the depletion of various antioxidants that combat free radicals, including glutathione peroxidase, catalase, and superoxide dismutase (109). This leads to a situation of oxidative stress, which has been associated with stress as well as the etiology of numerous disease conditions. As reactive free radicals’ peroxidation of membrane lipids is one of their primary side effects and polyunsaturated fatty acids are abundant in the encephalic areas, this process causes significant impairment to the structure and functionality of biological synapses (110). Thus, it was hypothesized that the primary biochemical alteration and outcome of oxidant-induced neuronal injury—lipid peroxidation—was what led to the emergence of neurobehavioral disorders like anxiety.

Proteins, lipids, and DNA are all damaged by high concentrations of NO and its byproducts. Direct interactions between nitric oxide and ROS can result in reactive nitrosative stress. A key process in the synthesis of RNS is the reaction between NO and superoxide anions, which results in the formation of the strong neurotoxin peroxynitrite (ONOO-), a highly reactive metabolite that may be harmful to a number of cellular constituents as well as macromolecules (111). The synthesis of adenosine triphosphate may change as a result of NO and peroxynitrite damaging nearly all of the mitochondrial respiratory chain’s elements. In particular at complex IV (also known as cytochrome c oxidase), these RNS reversibly or irreversibly impede mitochondrial oxygen consumption, which may cause cellular energy deficiency and ultimately cell death in pathological situations. The most vulnerable mitochondrial target for RNS is cytochrome c, and it is now known that NO and peroxynitrite inhibition of cytochrome c oxidase culminates in neuronal dysfunction as well (112). Further, NO has been demonstrated to alter the potential of the mitochondria in neuronal cells, which culminates in the opening of the mitochondrial permeability transition pore (MPTP). The exchange of solutes and a number of small proteins between the mitochondrial matrix and the cytosol can be ascribed to MPTP opening. These events may cause mitochondria to enlarge, the outer mitochondrial membrane to rupture, caspases to become activated, and the commencement of neuronal apoptotic pathways (113).

4.4. Limitations

The following restrictions—many of which have to do with confounding—should be taken into consideration when interpreting our findings. For instance, there is no consideration of other factors related to anxiety, such as antioxidant markers (super oxide dismutase, catalase, and their association with other factors such as sleep disorders or underlying medical conditions). Also, other cofounders are comorbid diseases and drugs that participants were taking during the study.

To determine statistical significance and to identify subtle changes, it is important to consider the sample size. Lack of statistical significance for clinical outcomes that appear to be significant could be due to insufficient sample size (114). Of the three cross-sectional studies included in the analysis, two had a small total sample size (n < 75) (43, 50) and one a very small sample size (n < 20) (51). The small sample sizes increase subject random variability, which may be the cause of conflicting outcomes among research. Interventional studies are required to more clearly show a cause-effect relationship; the majority of the studies cited above lack randomized, blinded, placebo-controlled trials. The third drawback is that different evaluation criteria and methodologies were used in the analyzed studies, making it possible for bias to be introduced into the evidence of convergence.
The results of these studies, although they fall under the same classification, cannot be compared because of their dissimilar natures, study populations, and study designs. Future studies should take into account a variety of variables that affect NO generation and metabolism, as well as potential therapies. Likewise, anxiety disorders are sometimes regarded as comorbid diseases. Future studies should take more notice of how other factors influence the implications of the findings; it is therefore imperative to analyze participants to determine if they are suffering from other mental illnesses.

5. Conclusion

The focus of this discussion was whether NO is associated with anxiety disorders. This link seems logical, yet the results of our analyses by and large do not provide convergence evidence for the involvement of NO in anxiety disorders in humans. Although there is currently little human data on which to base this conclusion, there are methodological and practical constraints that might have prevented a valid assessment of the associations of interest. Future research should focus on minimizing confounding and measuring error to better understand the potential involvement of NO in the pathophysiology of anxiety disorders in humans. Among animals, most but not all studies reported anxiogenic behavior of NO; and also, to reach a coverage notion of the role of NO in anxiety, minimizing confounding and errors is needed for future studies.

Abbreviations

NO : Nitric Oxide; NOS : Nitric Oxide Synthase; ROS : Reactive Oxygen Species; PNS : Peripheral Nervous System; CNS : Central Nervous System; VNTR : Variable Number Tandem Repeat; ex1f : Exon 1f; PD : Panic Disorder; HPA : Hypothalamic–pituitary–adrenal; GAD : Generalized Anxiety Disorder; ACTH : Adrenocorticotropic Hormone; CRH : Corticotropin Releasing Hormone; AVP : Arginine Vasopressin; CSAI-2 : Competitive State Anxiety Inventory-2; CIDI : Composite International Diagnostic Interview; SSAI : Spielberger State Anxiety Inventory; HAM-A : Hamilton Anxiety Rating Scale; CBT : Cognitive Behavioral Therapy; BPAD : Bipolar Affective Disorder; NF-κB : Nuclear Factor Kappa B; TNFα:
Tumor Necrosis Factor-alpha; **SAS**: Sympathoadrenomedullary System; **PVN**: Paraventricular Nucleus; **GRs**: Glucocorticoid Receptors; **MPTP**: Mitochondrial Permeability Transition Pore.

**Declarations section:**

**Acknowledgments:** I would like to thank all the scholars who have carried out previous studies to pave the way for gaining our knowledge, irrespective of the subject.

**Ethical Approval and Consent to Participate:** This research was a review, and no participants took part, so there was no need for it.

**Authors’ Contributions:** This article has one author who initiated the A.A. He has completed all aspects of this article.

**Availability of Data and Materials:** All data and materials are within the paper.

**Funding:** This study did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

**Competing Interests:** There is no competing interest to be declared.
References


107. Walker DL, Davis M. Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus


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**Appendix 1.**

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