



# The role of brain gaseous neurotransmitters in anxiety

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## Abstract

Although anxiety is perhaps one of the most significant current medical and social problems, the neurochemical mechanistic background of this common condition remains to be fully understood. Multifunctional regulatory gasotransmitters are novel, atypical inorganic factors of the brain that are involved in the mechanisms of anxiety responses. Nitric oxide (NO) signaling shows ambiguous action in animal models of anxiety, while NO donors exert anxiogenic or anxiolytic effect depending on their chemical structure, dose, treatment schedule and gas release rapidity. The majority of NO synthase inhibitors act as a relatively potent anxiolytic agents, while hydrogen sulfide (H<sub>2</sub>S) and carbon monoxide (CO) delivered experimentally in the form of “slow” or “fast” releasing donors have recently been considered as anxiolytic neurotransmitters. In this comprehensive review we critically summarize the literature regarding the intriguing roles of NO, H<sub>2</sub>S and CO in the neuromolecular mechanisms of anxiety in the context of their putative, yet promising therapeutic application. A possible mechanism of gasotransmitter action at the level of anxiety-related synaptic transmission is also presented. Brain gaseous neuromediators urgently require further wide ranging studies to clarify their potential value for the current neuropharmacology of anxiety disorders.

**Keywords** Nitric oxide · Hydrogen sulfide · Carbon monoxide · Anxiety

## Introduction

Anxiety disorders, as the most common mental dysfunctions, are a serious and growing medical and social problem [1]. Despite thousands of investigative studies, the complex nature of the neurochemical basis of anxiety is still not fully understood. The fundamental roles of anxiety acquisition, its origin and manifestations involve specific neural circuits of the amygdala, dorsolateral prefrontal cingulate cortex (DLPFC), ventromedial anterior cortex and hippocampal formation [2, 3]. A wide range of neurotransmitters and neuromodulators have been demonstrated to be involved in the

pathogenesis of anxiety disorder [4]. The key inhibitory neurotransmitter of the brain, *g*-amino butyric acid (GABA), has long been regarded as the most important factor in the etiology of anxiety, with GABA receptors being the main targets of anxiety related therapeutics such as benzodiazepines [5]. Stimulation of the GABA<sub>A</sub> receptor has been associated with anxiolytic activity, whereas its inhibition may trigger anxiety responses. The expression of several enzymes responsible for neural steroidogenesis, such as 5 $\alpha$ -reductase, 3 $\alpha$ -hydroxysteroid dehydrogenase and aromatase (3 $\alpha$ -HSD), have been detected in the limbic structures [6, 7], and an important role of glutamatergic neurotransmission at the level of hippocampal and amygdalar networks is widely postulated [8–10]. Serotonin and dopamine signaling, especially within the raphe nuclei, *locus coeruleus*, hippocampus and amygdala, are shown to be positively involved in the pathogenesis of anxiety [11–13]. Noteworthy, cholecystokinin (CCK)-immunoreactive circuits and CCK2 receptors within brainstem centers may play a role in anxiety responses [14]. Neuropeptide S (NPS), urocortins and the newly identified regulatory neuropeptides nesfatin-1 and phoexinin are recently considered as potent anxiolytic factors [15, 16]. Also, adenosine acting through A1 and A2A receptors may

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exert anxiolysis via stimulation of GABA exocytosis in hippocampal formation [17].

The widely studied multifunctional gaseous neurotransmitters: nitric oxide (NO), hydrogen sulfide (H<sub>2</sub>S) and carbon monoxide (CO) are small regulatory molecules of the brain that offer a novel interest. These inorganic neuromodulators are thought to be new factors involved in the mechanisms of anxiety pathogenesis. It is interesting to postulate that the famous eighteenth century discoverers of these harmful gases Joseph Priestley (1772, NO), Carl W. Scheele (1777, H<sub>2</sub>S) and William Cruickshank (1800, CO) could envision a future in which these gases would be considered important molecules which play a key role in several brain functions. NO, CO and H<sub>2</sub>S are commonly grouped as a family of signaling molecules called gasotransmitters [18]. They have specific cellular and molecular targets and are involved in signal transduction and modulation of metabolic systems, in both physiological and pathological conditions [19, 20]. These molecules are strongly lipophilic and easily soluble, having low molecular weight, short half-life, and easily able to cross cell membranes. Different to traditional neurotransmitters, gasotransmitters are synthesized only when required, importantly avoiding the necessity of vesicle storage. They can also be released from any part of the neural or glial cell and transmission can occur in non-classical directions, for example from the postsynaptic to the presynaptic neuron or to other nearby postsynaptic cells [18, 21]. All known gasotransmitters are able to diffuse readily through the cellular lipid bilayer and do not require typical membrane receptor binding or synaptic mechanisms to initiate signaling. Just like classical neurotransmitters the effects of endogenously synthesized NO, H<sub>2</sub>S and CO can be mimicked by the same compounds or via their specific donors administered experimentally. All three have well defined targets in neurons or glial cells and usually act via activation of a secondary messenger (cGMP, cAMP) cascade [22, 23]. Importantly, NO, H<sub>2</sub>S and CO have an interdependence concerning both their own availability and that of O<sub>2</sub> [18, 24]. Therefore, it is not impossible that other inorganic gas molecules such as nitrous oxide (N<sub>2</sub>O), sulfur dioxide (SO<sub>2</sub>), carbon suboxide (C<sub>3</sub>O<sub>2</sub>) [25–30] or even hydrogen cyanide (HCN) [31] can play a role of endogenous neuromodulators in some brain structures.

Currently, we have witnessed an increase in anxiety disorders hand in hand with efforts to produce more effective medications than the traditional benzodiazepines, selective serotonin reuptake inhibitors (SSRI) and 5-HT<sub>1A</sub> partial agonists. The ideal drug would only reduce anxiety symptoms with limited common side effects of the aforementioned classical anxiolytics such as dependence and withdrawal, severe sedation, memory deficits or metabolic disturbances [32–34]. Alternative treatment strategies based on modulation of other signaling pathways e.g., nitric

and H<sub>2</sub>S and CO-related transmission are worth researching. Several recent studies on gasotransmitter donors and new compounds targeting NOS have shown quite promising results in animal models. Gasotransmitters are currently considered as new intriguing factors in the pathophysiology of neuropsychiatric dysfunctions including anxiety disorders. Forthcoming innovation of anxiety pharmacotherapy could potentially be based on the precise and selective modulation of NO, H<sub>2</sub>S and CO signaling. Therefore, wide ranging basic investigations are urgently needed to clarify the efficacy and safety of these promising treatment strategies.

## Nitric oxide in the CNS

Nitric oxide (NO) is a versatile messenger of both the central and peripheral nervous system which plays an important regulatory role in several neurochemical processes [32–34]. Brain-derived NO acts as a regulator of key functional events such as classical neurotransmitter release (e.g., serotonin, glutamate and GABA), adult neurogenesis, synaptic plasticity, neuroinflammation, cellular immunity, vascular tone, and behavioral modulation [34–39]. NO can also modulate the synthesis and release of some multifunctional neuromodulators e.g., adenosine and ATP [40]. Activation of postsynaptic glutamatergic NMDA and AMPA receptors causes postsynaptic density protein 95 (PSD95)-related stimulation of neuronal nitric oxide synthase (nNOS). This key enzyme may also interact with the C-Terminal PDZ Domain Ligand of Neuronal NO Synthase (CAPON) and Dexamethasone-induced Ras-related protein 1 (DexRas1) to trigger MAP kinase signaling (MAPK). A blockage of nNOS-CAPON signaling transmission in mice with selective disruptors ZLc-002 or Tat-CAPON12C reversed chronic mild stress-induced anxiety behavior in elevated plus maze (EPM), open field (OF) and light–dark (LD) tests. Interestingly, this kind of inhibition increased synaptogenesis and dendritic rearrangement in cultured neurons isolated from the hippocampi of stressed animals probably via the cAMP response element-binding protein (CREB)-related pathway [41]. NO molecules cross the synaptic cleft and enter into the presynaptic neuropil where it activates soluble guanylyl cyclase (sGC) to produce cyclic cGMP (cGMP), which is able to affect neurotransmitter release machinery and to modulate the opening of ion channels: potassium ATP-gated (K<sub>ATP</sub>), potassium voltage-gated (VDKC) and cationic cyclic nucleotide-gated (CNG). From the structural viewpoint GC-coupled NO receptors are heterodimers made up of a common β1-subunit together with either an α1- or α2-subunit. Importantly, the α2-subunit contains a domain that allows binding of the α2β1 isoform to protein PDZ and finally targeting it to the synaptic regions [42]. Importantly, a stimulation of cGMP synthesis may exert several anxiolytic effects

and also extend benzodiazepine activity in animals (Fig. 2). NO binds to the heme group of the GC and most of the known effects of NO are due to activation of GC and the production of guanosine 3',5'-cyclic monophosphate (cGMP), however there are several brain signaling events independent of GC activation [43]. One important alternative is S-nitrosylation of thiol groups of several neural proteins [44]. NO can also bind oxygen to form dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>) which may attack thiol groups in the process called nitrosation [45].

It was recently reported that inosine monophosphate (IMP), which is a precursor of inosine, decreases nNOS levels in the rat cerebellum and ventral but not dorsal hippocampus. The phosphorylation of the cAMP response element-binding protein (CREB) was also increased in the ventral hippocampus and negatively correlated with nNOS expression, yet no changes in the neocortical nNOS activity were detected [46]. To conclude, NO acts as a multifunctional neuromodulatory molecule that can affect a number of signaling pathways which are involved in the mechanism of anxiety [47]. It should be emphasized that highly elevated NO concentrations may often exert diverse neurotoxic or even neurodegenerative effects such as oxidative stress reactions and induction of the apoptotic cascade [48]. An overexpression of NOS and excess of NO production may trigger neuroinflammatory processes that affect neurotransmission and other anxiety-related aspects of neuronal functions. Recent reports show that oxidative injuries are often caused by the generation of peroxynitrite (ONOO) from superoxide radicals [49, 50].

## NO synthesis and metabolism

Nitric oxide is produced via L-arginine by nitric oxide synthase (NOS), which exists in three distinct isoforms: neuronal nNOS (*NOS 1*), inducible iNOS (*NOS 2*), and endothelial eNOS (*NOS 3*). nNOS and eNOS are regulated by intracellular calcium levels (iCa<sup>2+</sup>) but iNOS is considered a calcium-independent enzyme. A recent study suggests interestingly, that the short allele of a functional promotor polymorphism of *NOS1* (*NOS1* ex1f-VNTR) may be associated with higher anxiety and altered fear conditioning in the human amygdala and hippocampus [51]. Several experimentally applied NOSs inhibitors are currently known, such as *N*(ω)-nitro-L-arginine-methyl ester (L-NAME), NG-methyl-L-arginine acetate (L-NMMA)—non selective, preferentially eNOS blockers, *N*<sup>6</sup>-(1-iminoethyl)-L-lysine (L-NIL) selective iNOS inhibitor, *N*(ω)-propyl-L-arginine (L-NPA) and 3-bromo-7-nitroindazole (3-Br-7-NI)—highly selective nNOS blockers. The initial step in the neural biotransformation of this gasotransmitter is a synthesis of S-nitrosothiols via binding NO molecules to activated thiols. However,

nitrites and nitrates are the main oxidative metabolites of NO and the ratio of both compounds seems to be balanced by tissue redox status and therefore plasma nitrite concentration may reflect local eNOS activity and endothelial NO formation; while nitrate levels allow estimates of general N<sub>2</sub>/NO turnover [52]. At present numerous NO donors both classical, such as sodium nitroprusside, and new, e.g., several synthetic compounds (diazoniumdiolates, S-nitrosothiols) often caged inside metallic/silica or lipid nanoparticles (dendrimers and micelles) are considered to be the promising and innovative agents in experimental cancer treatment [53, 54].

## L-Arginine: a precursor of NO biosynthesis and anxiety

Short term (4 days) oral L-arginine (Arg) and L-lysine (Lys) administration at doses of 200 mg/kg (twice daily) reduced anxiety in male rats subjected to restraint stress immediately after treatment, while plasma corticosterone levels following the elevated plus maze (EPM) test was also significantly decreased [55]. Noteworthy, a similar pattern of anxiolytic changes including reduced plasma cortisol levels has also been observed in stressed pigs fed with an Arg/Lys fortified diet [56]. An effect of Arg/Lys dietary supplementation on anxiety states was also studied in humans, with a week long treatment with aminoacids (at dose 2.6 g/day) decreasing stress-induced anxiety, reducing concentrations of salivary chromogranin-A (a marker of adrenal sympathetic activity) and cortisol in Japanese male participants [57]. These results are in line with a previous report showing anxiolytic effects of 10 days-long Arg/Lys treatment in healthy subjects with relatively elevated trait anxiety exposed to a psychosocial stressor in the form of a public speech [58]. It is worth noting that in all aforementioned studies neither NO levels nor NOS activity were estimated. Thus, although Arg is the main precursor molecule in the NO synthesis there is no direct proof that the anxiolytic action of this amino acid is a result of increased gasotransmitter action. On the other hand, mice treated with L-arginine did not show any behavioral alterations in the EPM test but administration of this amino acid in combination with sildenafil, a phosphodiesterase 5 inhibitor, caused a significant anxiogenic effect probably through the activation of a NO-cGMP signaling pathway [59].

## Exogenous NO donors and anxiety

It should be emphasized that the number of reports dealing with the effects of NO donors in behavioral animal models of anxiety are often contradictory or ambiguous (Table 1). An injection of NOC-9, a NO donor, into mice bed nucleus of the stria terminalis (BNST), a limbic structure with abundant

**Table 1** Summary of the main studies dealing with the relationships between gaseous neurotransmitters and anxiety

Substance	Dose	Route	Species	Effect	Methodology	References
NO precursor						
L-Arg	200 mg/kg daily	p.o. for 4 days	Rat	Anxiolytic	EPM test	[55]
L-Arg	a.l. for 10 days	Arg/Lys fort diet	Pig	Anxiolytic	Inactivity time test	[56]
L-Arg	2.6 g/day	p.o. for a week	Human	Anxiolytic	STAI inventory	[57]
L-Arg	1, 5, and 10 µg/rat	Intra amyg. inj	Rat	Anxiolytic	EPM test	[90]
L-Arg	3 g/day	p.o. for 10 days	Human	Anxiolytic	STAI inventory	[58]
L-Arg	0.4, 0.8 µg/mouse	Intra hipp. inj	Mouse	Anxiogenic	EPM test	[89]
L-Arg + sildenafil	200+0.05–10 mg/kg	Acute, i.p	Rat	Anxiogenic	OF test	[59]
NO donors						
Molsidomine	1, 2, 4 mg/kg	Acute, i.p	Rat	Anxiolytic	LDB and OF tests	[62]
SNP	1, 3 mg/kg	Acute, i.p	Rat	Anxiolytic at 1 mg/kg	LDB test	[64]
SIN-1	0.1, 0.3, 1 mg/mouse	Acute, i.c.v	Mouse	Anxiolytic at 0.3, 1 mg/mouse	LDB test	[63]
SNP	0.1, 0.3 and 1 mg/kg	i.p. for 5 days	Rat	Anxiolytic	LDB and OF tests	[65]
NOC-9	18.7, 37.5 or 75 nmol	Intra BNST inj	Mouse	Anxiogenic	EPM test	[60]
SNP	9.3, 18.7 or 37.5 nmol	Intra PFC inj	Mouse	Anxiogenic	EPM test	[61]
SNP	1, 2 and 3 mg/kg	Acute, i.p	Mouse	Anxiogenic at 3 mg/kg	Marble burying test	[66]
NOS inhibitors						
Aminoguanidine	50, 100, 150 mg/kg	i.p. for five weeks	Rat	Anxiolytic	EPM and OF test	[74]
L-NAME	10 mg/kg	i.p. acute or for 15 days	Rat	Anxiolytic	EPM and FST test	[79]
L-NAME/7-NI	50–200 and 5–10 nmol	Intra amyg. inj	Rat	Anxiolytic	EPM and LDB test	[80]
L-NAME	200 nmol	Intra PAG inj	Rat	Anxiolytic	LDB test	[82]
L-NAME/7-NI	10 mg/kg	Acute, i.p	Rat	Anxiolytic	EPM test	[88]
L-NAME	1, 5 and, 10 µg/rat	Intra amyg. inj	Rat	Anxiolytic	EPM and FST tests	[90]
NPLA	0.04 nmol	Intra MPFC inj	Rat	Anxiolytic	EPM test	[91]
L-NOARG	20 and 40 mg/kg	Acute, i.p	Mouse	Anxiolytic	EPM test	[87]
7-NI	20–120 mg/kg	Acute, i.p	Mouse	Anxiolytic	EPM test	[87]
L-NAME	10, 25, and 50 mg/kg	Acute, s.c	Mouse	Anxiogenic	EPM and LDB tests	[78]
L-NOARG	10 mg/kg	Acute, s.c	Mouse	Anxiogenic	EPM test	[83]
L-NOARG	10 mg/kg	Acute, s.c	Mouse	Anxiogenic	EPM test	[84]
L-NOARG	30–120 mg/kg	Acute, i.p	Rat	Anxiogenic	EPM test	[85]
L-NOARG	0.5, 1, 2 or 4 µg/µl	Intra hipp. and amyg	Rat	Anxiogenic	EPM test	[86]
L-NAME	20 and 40 mg/kg	Acute, i.p	Rat	Anxiogenic	EPM test	[87]
L-NAME	40 ng/animal	Intra hipp. inj	Rat	Anxiogenic	EPM test	[89]
H <sub>2</sub> S donors						
Na <sub>2</sub> S	4, 8 and 12 mg/kg	i.p. for 8 days	Rat	Anxiolytic	OFT test	[114]
NaHS	3 and 5 mg/kg	i.p. for 11 weeks	Rat	Anxiolytic	EPM, MWM tests	[115]
NaHS	1.68 or 5.6 mg/kg	i.p. for a week	Rat/Mse	Anxiolytic	EPM and FST tests	[113]
CO donors						
Heme lysinate	600 nmol	Intra LC inj	Rat	Anxiolytic	EPM and LDB tests	[127]
CORM-2	5 mg/kg	i.p. for 10 days	Rat	Anxiolytic	EPM and LDB tests	[133]
CORM-3	4 mg/kg	i.v. (femoral vein) in TBI	Rat	Anxiolytic	EPM and OF tests	[134]
SO <sub>2</sub> donors						
Na <sub>2</sub> SO <sub>3</sub> /NaHSO <sub>3</sub>	5, 20, 50, 100 mg/kg	Acute, i.p	Mouse	Anxiolytic	OFT test	[137]

*amyg.* Amygdala, *BNST* bed nucleus of the stria terminalis, *CORM-2* tricarbonyldichlororuthenium [II] dimer, *CORM-3* ruthenium(II) complex Ru(glycinato)Cl(CO)<sub>3</sub>, *EPM* elevated plus maze test, *FST* forced swimming test, *hipp.* Hippocampus, *i.p.* intra peritoneal injection, *i.v.* intravenous injection, *LC* locus coeruleus, *LDB* light–dark box test, *MPFC* medial prefrontal cortex, *L-NAME* *N*(ω)-nitro-L-arginine-methyl ester, *L-NOARG* NG-nitro-L-arginine, *7-NI* 7-nitroindazole, *NOC-9* 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine, *NPLA* *N*-propyl-L-arginine, *OF* open field test, *PAG* periaqueductal gray, *PFC* prefrontal cortex, *p.o.* oral treatment, *s.c.* subcutaneous injection, *SIN-1* 3-morpholiniosyndnoimine, *SNP* sodium nitroprusside, *STAI* state-trait anxiety

expression of glutamatergic, corticotrophin releasing factor (CRFergic) and nitrenergic perikarya, resulted in anxiogenic effects in the EPM test. Interestingly, a previous intra-BNST injections of CP376395, a CRF type 1 receptor antagonist (CRF1), or AP-7, an NMDA (*N*-methyl-*D*-aspartate) attenuated the anxiety promoting effects of administered NO-donor, suggesting that CRF1 and glutamatergic signaling differentially affect NO-induced aversive behavior in the mouse BNST [60]. An acute microinjection of NOC-9 (at dose 9.4–37.5 nmol) into the mouse medial prefrontal cortex (mPFC) caused anxiogenic effect evidenced in the EPM test [61]. Conversely, an intraperitoneal administration of molsidomine, a novel NO donor induced a significant anxiolytic effects in rats in the open field (OFT) and light/dark box (LD) tests, that was notably not different to that evoked by diazepam, (a GABA agonist) treatment [62]. Intracerebroventricular infusion of the next NO donor morpholin-syndnonimine (SIN-1) at doses 0.3–1 mg/kg also resulted in anxiolytic effect in mice in the LD test [63].

Sodium nitroprusside (SNP), an inorganic fast NO donor in the form of ruby, water soluble crystals may regulate anxiety responses in rats under condition of the light/dark box (LD) test, although this has been shown to be dose, time and treatment schedule-dependent [64]. SNP administered 30 min before testing and not infused for 30 min, induced anxiolytic-like behavior, which could not be attributed to changes in locomotor activity. While repeated application of SNP (1 and 3 mg/kg, for 5 consecutive days) did not alter rodent behavior in both LD and motor activity tests. The authors underlined that SNP presents a narrow therapeutic window and may release potentially toxic products during its decomposition [64]. Papageorgoulis et al. [65] observed that rats subchronically treated with SNP (0.1, 0.3 and 1 mg/kg) for five consecutive days, presented a decrease in anxious behavior, whereas 0.1, 0.3 and 1 mg/kg SNP, 10 min or 30 before the test, did not exert significant modification of animals behavioral paradigms. On the other hand, intraperitoneal injection of SNP (dose 1–3 mg/kg) increased anxiety-like compulsive behavior in mice [66]. A complete mechanistic explanation of all the aforementioned contradictory reports remains elusive. Possibly, anxiogenic/anxiolytic effects of NO donors are strictly dose dependent. On the other hand NO may affect release of diverse anxiety-related both excitatory and inhibitory neurotransmitters e.g., serotonin and GABA [67, 68].

## NOS inhibitors and anxiety

Several basic studies have shown that inhibition of NO synthesis might produce anxiolytic and/or antidepressant-like effects [37, 69–71]. Almost 10 years ago Karolewicz et al. [72] found that blockade of NOS decreases serotonin

turnover in the mouse frontal cortex, similar to the effect of imipramine, while a more recent report [73] showed that fluoxetine downregulates expression of nNOS in the hippocampus. Beheshti et al. [74] assessed the effect of iNOS inhibition by aminoguanidine on the development of anxiety- and depression-like behaviors using elevated plus EPM, OFT, and forced swimming tests (FST) following LPS-induced inflammation in rats. The authors reported a clear relationship between oxidative stress and behavioral disturbances, with the overproduction of NO followed by increased iNOS activity and anxiety-like behavior. Aforementioned nonselective nitric oxide synthase inhibitors (*L*-NAME, *L*-NMMA and 7-NI), act by attenuating adrenocorticotropin hormone (ACTH) responses to electric shocks [75–77], further linking NO to anxiety-related symptoms. An important study by Czech et al. [78] investigated the effects of NOS inhibition with *L*-NAME on anxiety related behavior in mice using EPM and LD tests. A lack of differences between *D*-NAME (an inactive NAME isomer) and vehicle in their actions may prove that NOS blockers act in a stereospecific manner. Some dose-related decrease was observed in the EPM test however, the effect was relatively weak and rather unclear. On the other hand, LD tests showed an anxiogenic-like action of NOS blockade, suggesting anxiolytic properties of NO [78]. However, the majority of studies report anxiolytic effect of NOS inhibition in animal models. For instance, NOS blockade with *L*-NAME strongly prevented long-term stress-induced anxiogenesis in rats in the EPM and FST tests [79], while an analogous anxiolytic effect of bilateral *L*-NAME and 7-NI microinjections to the rat medial amygdala (MeA) was reported [80]. Importantly, the effect of *L*-NAME administration was prevented by treatment with NO donor *L*-arginine. Also aminoguanidine (AG) and 7-NI caused anxiolytic effect in stressed but not unstressed mice under EPM and LDT tests. The level of nitrites was also elevated in stressed rodents exclusively and it was attenuated by AG but not 7-NI administration. Of note, all effects of AG were augmented by pyrrolidinedithio-carbamate (PDTTC), an inhibitor of NF-kappaB induction, in stressed animals, suggesting an involvement of NOS in stress-induced anxiogenesis [81]. It was also recently reported that NO signaling in the periaqueductal gray (PAG) nucleus may play a role in the regulation on ethanol withdrawal-induced anxiety behavior of rats. Treatment with *L*-NAME caused anxiolytic effects in these animals in the LD test, whereas *L*-arginine abolished the action of NOS inhibitor [82].

In contrast, a number of early studies reported anxiogenic-like effects NOS inhibition. For instance, injection of *NG*-nitro-*L*-arginine (*L*-NOARG) abolished anxiolytic activity of chlordiazepoxide [83] and nitrous oxide [84] in mice in EPM test. Acute intraperitoneal administration of *L*-NOARG [at doses 30–120 mg/kg] caused anxiogenic effects in rats



in the EPM test [85]. This action was not observed after 4 days-long L-NOARG treatment (at dose 3.75–60 mg/kg, twice a day). Moreover, it was blocked by i.c.v. injection of L-arginine (dose 1000 nmol). A decrease in the time spent by rats in the EPM test was also reported after targeted infusion of L-NOARG to the amygdala and hippocampus [86]. The systemic administration of L-NAME (20.0 and 40.0 mg/kg) caused anxiogenic effect in unstressed control mice but 7-NI (20.0–120.0 mg/kg) and L-NOARG (20.0 and 40.0 mg/kg) induced anxiolysis in the EPM test. Importantly, in stressed animals (small platform model), the 7-NI at a dose of 20.0 mg/kg caused in turn an anxiogenic effect, other doses of this selective nNOS inhibitor, with exception of 80.0 mg/kg, as well as L-NOARG and L-NAME did not change the rat behavior. Possibly, some alterations in the brain NOS-NO pathways as well as central action of NOS inhibitors may be stress-related [87]. Stressed rats pretreated with L-NAME or 7-NI (both at doses 10 mg/kg) exhibited distinct attenuation of anxiety responses in the EPM test. Additionally, administration of this NOS blocker reversed stress-induced increase in corticosterone and NO derivatives (NO(x)) in plasma. 7-NI, but not L-NAME, reversed stress-induced NO(x) in the hypothalamic paraventricular nucleus (PVN) and locus coeruleus (LC). Collectively, this may suggest that L-NAME affects HPA axis activity, while 7-NI may exert anxiolytic-like effects via reduction of NO(x) level in several brain structures e.g., ventromedial prefrontal cortex; vMPFC [88]. Injection of L-NAME (40 ng/animal) and L-arginine (0.4 and 0.8 µg/animal) into the mouse dorsal hippocampus induced anxiogenic responses. Both substances abolished the anxiogenic effect of histamine and pyrilamine infusion (9 mg/mouse). Furthermore, intra-CA1 administration of L-NAME and L-arginine caused anxiogenic effects [89]. Nitroergic neurons of the rat basolateral amygdala (BLA) are recently considered to play an important role in stress-dependent anxiety and depression. Stress-induced anxiety measured with the EPM test was reduced by intraamygdalar infusions of L-arginine and L-NAME (1, 5, and 10 µg/rat). Noteworthy, both agents, in all doses showed the same anxiolytic effect [90]. The anxiogenic-like effect of restraint stress evaluated with EPM test was also reversed by the injection of *N*-propyl-L-arginine (NPLA) at dose 0.04 nmol into the rat prelimbic region of vMPFC [91].

The background literature also brings contrasting results dealing with the relationship between NO signaling and anxiety in animal models (Table 1). The anxiolytic, dose dependent effects of L-arginine, NOS blockers: L-NAME, 7-NI and 8-Br-cGMP (a cGMP analogue) microinjections into the rat dorsal raphe nucleus (DRN) are reported [92]. Of note, while L-NAME and 7-NI at low doses caused anxiolytic-like effects, higher doses decreased locomotor activity. On the other hand, 8-Br-cGMP (25 and 50 nmol) increased the number of closed arm entries but did not

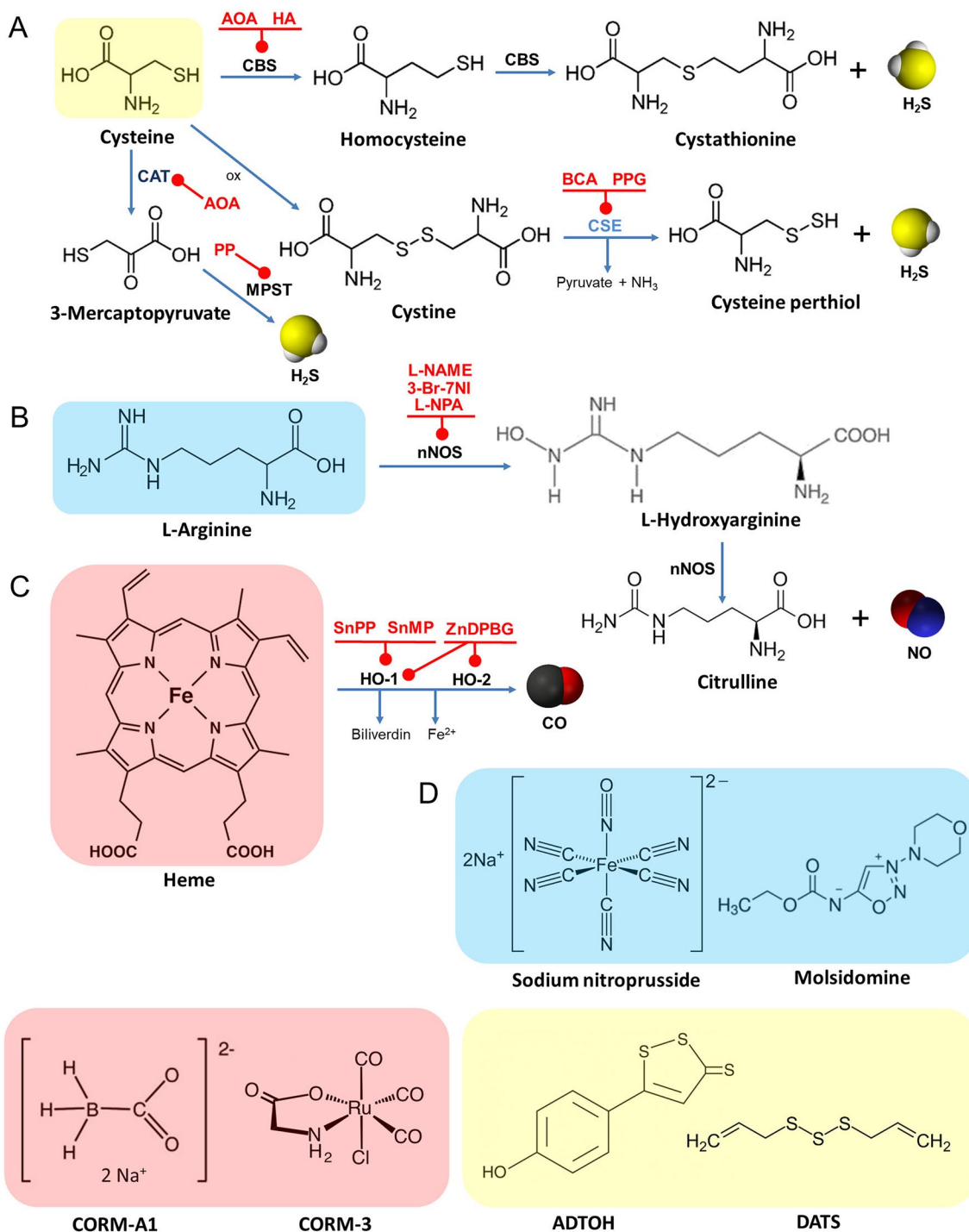
produce changes in anxious behaviors measured with the EPM test. Dual effects on anxiety following interference with NO-mediated neurotransmission were reported [92]. Corroborating to the existing duality, Pitsikas [38] pointed out that, it was not possible to conclude whether activating NO pathways leads to increased or reduced anxiety-like behavior. Furthermore, it is also highlighted that there is no information regarding the implication of eNOS and iNOS on psychiatric disorders nor is it clarified if the effects of NO on anxiety are sex-dependent or not.

## Brain-derived hydrogen sulfide

Hydrogen sulfide (H<sub>2</sub>S) is commonly known as a toxic, colorless gas with the characteristic obnoxious odor of rotten egg proteins. The majority of current reports unambiguously suggest that the H<sub>2</sub>S molecule should be attached to the family of gaseous neurotransmitters in addition to nitric oxide and carbon monoxide [93–96]. In the CNS, H<sub>2</sub>S is involved in several processes facilitating the long-term potentiation (LTP) and upregulation of the GABA<sub>B</sub> receptor in the hippocampus [97, 98]; modulation of the hypoxic ventilator response; regulation of intracellular calcium homeostasis [99], pH balance in neuronal and glial cells [99] and neuroprotection against oxidative stress [100]. Sodium hydrosulfide (NaHS) injection decreased hippocampal impairment in the rat brain after cerebral ischemia [101], but gaseous H<sub>2</sub>S reduced the neurological damage in the same experimental condition of common carotid artery occlusion [102]. However, it should be emphasized that in the animal model of stroke only low doses of NaHS exerted a neuroprotective effect, with higher ones being neurotoxic [103]. Furthermore, it was reported that Na<sub>2</sub>S may increase neuronal survival after ischemic injury of mouse cardiac muscle and animals with heart CSE overexpression had a better neurological outcome following the tape removal test [104]. Treatment with Na<sub>2</sub>S may also have beneficial behavioral effects in the rat model of cardiac arrest, however it does not affect neuronal apoptosis processes [105].

## Neural and glial H<sub>2</sub>S biosynthesis and metabolism

Hydrogen sulfide is synthesized in both neurons and astrocytes from L-cysteine (Cys) with the participation of three key enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and cysteine aminotransferase (CAT), Fig. 1. Catalytic activities of CBS and CSE are tissue specific [106]. Also 3-mercaptopyruvate sulfurtransferase (MPST) is involved in brain H<sub>2</sub>S synthesis from 3-mercaptopyruvate (3-MPT). Of note, an interesting crosstalk between



**Fig. 1** The main pathways of gas neurotransmitter biosynthesis in the brain with key regulatory enzymes and their exogenous inhibitors. Schematic representation outlines the origin of hydrogen sulfide (a), nitric oxide (b) and carbon monoxide (c) from the precursors (on different color backgrounds). All enzyme blockers are marked in red and round-ended lines represent their inhibitory effect. The molecular structures of selected exogenous donors of NO, H<sub>2</sub>S and CO are also shown (d). Anethole dithiolethione; ADTOH aminooxyacetate, AOA b-cyano-L-alanine, BCA 3-bromo-7-nitroindazole (3-Br-7-NI), 3-Br-

7NI cysteine aminotransferase, CAT cystathionine-b-synthase, CBS sodium boranocarbonate, CORM-A1 tricarbonylchloro(glycinato) ruthenium, CORM-3 cystathionine-g-lyase, CSE diallyl trisulfide, DATS hydroxylamine, HA heme oxygenases 1 and 2, HO-1/HO-2 N( $\omega$ )-nitro-L-arginine-methyl ester, L-NAME N( $\omega$ )-propyl-L-arginine, L-NPA mercaptopyruvate sulfurtransferase, MPST phenylpyruvate, PP propargylglycine, PPG tin mesoporphyrin, SnPP zinc deuteroporphyrin 2,4-bis glycol; ZnDPBG

NO and CO pathways may regulate heme moiety of CBS has recently been suggested [107–109]. A non-enzymatic reaction of H<sub>2</sub>S synthesis which is catalyzed by pyridoxal 5'-phosphate (vitamin B6) and iron may also occur in some tissues especially within erythrocytes [110]. The metabolic elimination of H<sub>2</sub>S is based on its oxidation to persulfide by mitochondrial sulfide quinone oxidoreductase (SQR), persulfide dioxygenase (ETHE1) and also by the methylation via the cytoplasmic enzyme cysteine dioxygenase (CDO) [111]. The further oxidation of persulfide by ETHE1 results in sulfite formation and finally sulfites are metabolized by sulfite oxidase or rhodanese (thiosulfate sulfurtransferase) to rhodanide and sulfate respectively. Probably, superoxide dismutase (SOD) may oxidize H<sub>2</sub>S to polysulfides [112]. A number of exogenous H<sub>2</sub>S donors are known, such as rapid-releasing alkaline sulfides (Na<sub>2</sub>S, NaHS) and slow liberating sulfur compounds e.g., S-memantine, dithioloethione (ADT) and diallyl trisulfide (DATC) [96].

### Sodium sulfide: a fast exogenous H<sub>2</sub>S donor and anxiety

Currently, some studies are investigating the effects of H<sub>2</sub>S in depressive- and anxious-like behaviors. Chen et al. [113] investigated the anxiolytic and antidepressant effects of NaHS (doses 1.68 or 5.6 mg/kg) in rats and mice. Applying EPM and forced swimming test (FST) protocols, they observed antidepressant and anxiolytic effects in mice, while only anxiolytic effects in rats. Significantly, either NaHS or imipramine, applied intraperitoneally for 7 days, has shown antidepressant effects when compared to saline. Likewise, Donatti et al. [114] observed anxiolytic effects of 8 day-long sodium sulfide (Na<sub>2</sub>S) administration (dosage 4, 8 and 12 mg/kg) in rats during OFT. Corroborating these results, Chen et al. [107] correlated the current literature highlighting the potential role of H<sub>2</sub>S on pathophysiology and management of anxiety disorders, and the role of H<sub>2</sub>S in neuroplasticity and oxidative stress regulation by decreasing reactive oxygen species. Increasing neuroplasticity would likely result from glutamatergic transmission via NMDA receptors, long-term potentiation, and within neuronal and glial Ca<sup>2+</sup> homeostasis maintenance. A new study by Habitabar et al. [115] reported that NaHS significantly reduced anxiety-like behavior and improved memory in rats treated with a high-fat diet (HFD). It is not yet clear how H<sub>2</sub>S can impact the brain mechanism of anxiety. Potentially, this may stimulate GABA-related transmission (Fig. 2) and maintain intracellular reduced brain glutathione to decrease oxidative stress. Alternatively, it may facilitate amygdalar or hippocampal neuroplasticity via NMDA-dependent signaling and/or modulate neural and glial Ca<sup>2+</sup> homeostasis [107]. In the case of glutamatergic neurons H<sub>2</sub>S activates

NMDA receptor similarly to NO. On the other hand, in the inhibitory GABAergic neuron H<sub>2</sub>S may stimulate presynaptic GABA transporter 1 (GAT1) that increases GABA reuptake and also directly modulates postsynaptic GABA receptors as well as K<sub>ATP</sub> potassium channels. An increase in synaptic GABA concentration promotes postsynaptic neuron silencing that may support anxiolytic effects. Astrocytes are considered as alternative source of H<sub>2</sub>S in the brain, with the gas molecules produced activating adenylyl cyclase (AC) of adjacent neurons and modulate NMDA receptor signaling via cyclic AMP (cAMP) signaling. H<sub>2</sub>S can also cause rapid calcium release from astrocytic endoplasmic reticulum and the activation of excitatory amino acid transporter (EAAT1) that increases synaptic glutamate level.

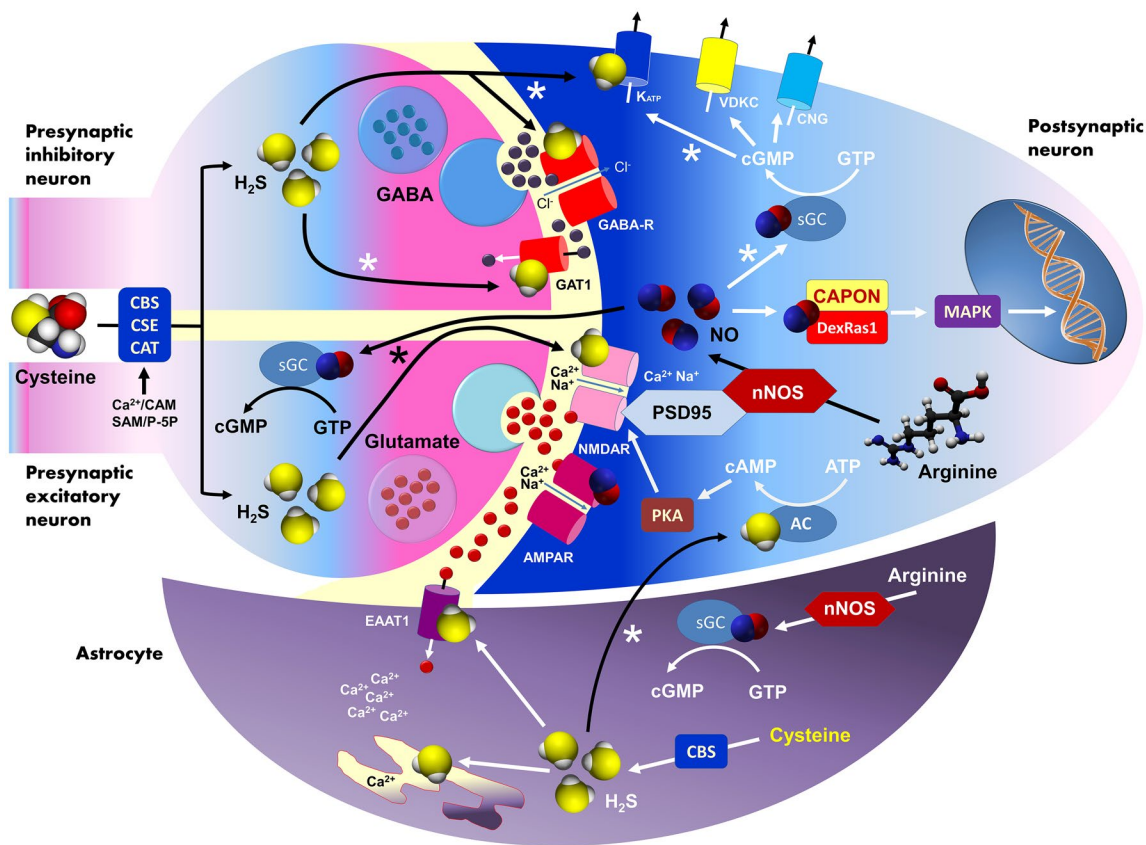
Anxiolytic effects in experimental, preclinical, and initial pharmacologic studies of H<sub>2</sub>S-releasing drugs such as S-diclofenac, natural sulfide compounds e.g., garlic and breathing retraining programs (which produce more available H<sub>2</sub>S) have also been described. A unique preclinical study reported intriguing functional effects of H<sub>2</sub>S on anxiety and fear extinction-related neurocircuits in the amygdala. Interestingly, breathing retraining programs, which are incorporated into tai chi and yoga, may increase plasmatic H<sub>2</sub>S, NO and CO molecules level and significantly reduce anxiety ratings in participants with hypertension [108, 116]. However, further studies are required to assess the potential applicability and innovational therapeutic actions of these techniques.

### Carbon monoxide in the brain

Carbon monoxide (CO) is commonly known as a highly toxic, haemoglobin deactivating and seriously hazardous industrial gas, a product of incomplete hydrocarbon combustion. However, CO is synthesized endogenously in several tissues where it can be involved in numerous biochemical and physiological processes such as signal transduction, blood pressure regulation, inhibition of myocytes proliferation, modulation of cell activity and maintaining of neural homeostasis and having an anti-inflammatory, anti-thrombotic and anti-apoptotic effect [22, 117]. In the brain CO activates sGC to increase cGMP stimulation of PKG. CO and NO bind sGC with similar affinity but CO-sGC is 25–50 times less active than NO-sGC [118]. CO may extend the opening time of calcium-gated large conductance potassium channels (BKCa) via binding to their heme domains [119, 120] and block inward rectifier channels (K<sub>ir</sub>) via an unknown mechanism [121]. Endogenous CO at normal temperature and pressure is chemically inert in the absence of d-electron metals [122].

There is also a suggestion that CO may affect synaptic turnover and signaling through the modulation of





**Fig. 2** Neurochemistry of nitric oxide and hydrogen sulfide in the context of anxiety-related cellular mechanisms. All molecular events potentially responsible for anxiolytic/anxiogenic properties of NO

and H<sub>2</sub>S were marked with asterisks. The figure is an original conceptual artwork partly based on data from selected references [37, 38, 98, 138]

dopamine and glutamate transporters. The modulation of cGMP synthesis by CO seems to be NO independent, structure specific and even sexually dimorphic e.g., exogenous CO increased striatal cGMP levels only in male individuals [123]. Since the early 1990s, CO therapeutic effects have been investigated as potential agents for treatments of diseases such as cancer, sepsis, hypertension, heart failure, inflammation, thrombosis, neurodegeneration and hematological diseases [23, 24, 124, 125]. However, the number of reports regarding potential effects of CO and its donors on anxiety is still scarce.

The possible forms of applying CO for both experimental and therapeutic purposes are inhaled gas, and safer CO releasing molecules (CORMs) that may be administered per os or parenterally. CORMs are unique boron (CORM-A1, a “slow” CO releaser) or heavy metals (CORMs 1–3) containing carbonyl complexes that aim to deliver, in a controlled way, limited amounts of CO to diverse tissues including brain structures [23, 125, 126] offering potentially therapeutic fine tuning of CO liberation.

### CO biosynthesis

Carbon monoxide (CO) is a gasotransmitter that stimulates guanylyl cyclase (GC) to form cGMP and acts by binding to iron atom at the active site of heme [117]. The endogenous production of CO occurs through the activity of heme oxygenase (HOCO, HOs) responsible for the cleavage of heme molecule into biliverdin, free iron (Fe<sup>2+</sup>) and CO. Two independent isoforms of this enzyme are known: an inducible heme oxygenase 1 (HO-1) and a constitutive form, heme oxygenase 2 (HO-2) [24]. A third probably inactive isoform HO-3 was cloned from rat brain tissue but its functions remains unknown [127, 128]. The expression of HO-1 is detected in the selective small cellular assemblies of the brain (both neural and glial) while distribution of HO-2 is rather ubiquitous [129]. Importantly HO-1 activity is upregulated by cellular stress suggesting that this enzyme represents the heat-shock protein family [103]. Relatively abundant HO-2 expression has

been found in the hippocampal formation, hypothalamus, neocortex, cerebellum and olfactory bulb [130, 131]. HO activities may be unspecifically blocked by clemizole derivatives or synthetic imidazoles e.g., azalanstat, but some kinds of tin porphyrin complexes (SnPP, SnMP) are considered as selective HO-1 inhibitors. Tissue-derived CO, too dilute to affect oxygen turnover is exhaled through the lungs [23].

## Exogenous CO donors and anxiety

One of the few pioneer studies on CO and anxiety was conducted by Carvalho-Costa et al. [127]. This important report showed that anxiolytic effect of CO in rats was evoked by cGMP-dependent heme oxygenase pathway in the rat locus coeruleus (LC), the main center of brain noradrenergic signaling [127]. Noteworthy, LC neurons show abundant HO-2/HO-1 expression suggesting a distinct role of CO-related mechanisms in their regulatory functions [132]. Targeted intra-LC microinjection of heme-lysinate (a substrate for HOs) significantly decreased anxiety symptoms during EPM and LDB tests. Conversely, microinjection of a selective sGC inhibitor (ODQ) prior to heme-lysinate abolished all aforementioned anxiolytic effects. On the other hand a treatment with zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), a nonselective HOs blocker did not alter animal behavior [127]. All neurochemical and physiological phenomena perceived in this investigation strongly suggest that the anxiolytic action of CO within rat *locus coeruleus* is strictly connected with local cGMP signaling. The aforementioned results were supported by a more recent study showing that CO, liberated both acutely (3 h) and chronically (10 days, twice a day) from an intraperitoneally administered tricarbonyldichlororuthenium [II] dimer (CORM-2) molecule promoted an anxiolytic-like effect (examined with EPM and LD tests) and increased HO-1 activity in the rat LC [133]. Additionally, both short- and long-term treatment with cobalt protoporphyrin IX, (CoPP), a HO-1 inducing complex resulted in the same behavioral effects. Treatment with CORM-3 significantly reduced anxiety-like behavior in rats after transitional brain ischemia (TBI), improved brain circulation and promoted neuronal survival in the amygdala [134].

By applying intracerebroventricular injections of tin protoporphyrin (SnPP, selective HO-1 inhibitor) and L-NAME, Kim and Rivier [77] were able to verify that NO and CO may exert a stimulatory influence in physicoemotional stressors (ACTH mediated responses) and that the hypothalamus is a critical site for the central regulatory actions of both gasotransmitters. The authors remarked that NO (and also CO) might exert opposite effects in the hypothalamic paraventricular nucleus (PVN) when compared to other

neuroendocrinal structures (median eminence and pituitary) [77]. The same research group had already found that there was a blockade of endogenous CO formation after systematic HO-1 blockers: SnPP and tin mesoporphyrin (SnMP) injections, followed by a reduction of plasmatic ACTH when rats were exposed to mild electroshocks [76].

## Sulfur dioxide and anxiety responses

Interestingly sulfur dioxide (SO<sub>2</sub>) synthesized during intracerebral amino acid metabolism has also been very recently suggested as a new gaseous transmitter [27]. Brain-derived SO<sub>2</sub> a product of aspartate aminotransferase (AAT) catalytic activity can easily pass through all neurolemmal barriers and is considered to be involved in some cardiovascular regulatory processes including blood pressure control [135]. This novel study reported that treatment with SO<sub>2</sub> significantly reduced neuronal injury in the rat hippocampus after chronic cerebral hypoperfusion (CCH) and improved cognitive disturbances in the Morris water maze. Of note, hippocampal catalase activity was also elevated suggesting that SO<sub>2</sub> may support brain antioxidant responses [136]. It was recently reported that treatment with exogenous SO<sub>2</sub> donors sodium sulfite and hydrosulfite (Na<sub>2</sub>SO<sub>3</sub>:NaHSO<sub>3</sub>) caused a distinct anxiolytic-like effect in mice (in the OFT test) under normal conditions and abolished chronic mild stress (CMS)-induced anxiety-like behavior. This may open a cautious discussion about SO<sub>2</sub> as a new possible agent for the treatment of anxiety [137, 138].

## Concluding remarks

A number of reports have clearly demonstrated that nitric oxide molecules are involved in the neurochemistry of anxiety responses in animal models and both NO donors and selective NOS inhibitors can be rightly considered in the future treatment of anxiety disorders. Moreover, recent findings have revealed that hydrogen sulfide and carbon monoxide may also exert distinct anxiolytic effects and its donors seem to be applicable. It should not be excluded that a novel way to treat anxiety has been postulated and may be an innovative alternative in the development of new therapeutic possibilities. However, there are still numerous concerns related to the pharmacological effects of gaseous neurotransmitter donors or modulators and further basic investigations are urgently required to prove their potential usefulness for the pharmacotherapy of anxiety. Firstly, all potential treatment schedules either acute or long-term should be precisely evaluated. Secondly, more detailed animal studies should assess the efficacy and safety-profile of NO, H<sub>2</sub>S and CO donors as anxiolytic factors using a large spectrum of behavioral

methods. Importantly, a relatively narrow therapeutic window of these substances as well as their potential biphasic actions should also be taken into account. To conclude, an alternative way to treat anxiety has potentially been identified and it may introduce a new therapeutic strategy in current neuropsychiatry. Nevertheless, there are still numerous questions related to the anxiolytic properties of gaseous neurotransmitters and more advanced pharmacological studies are needed to confirm their possible clinical applicability.

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