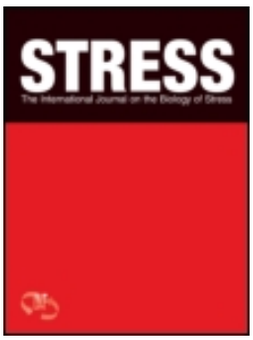

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The Roles of Carbon Monoxide and Nitric Oxide in the Control of the Neuroendocrine Stress Response: Complementary or Redundant

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There is widespread evidence in favour of nitric oxide (NO) acting as a gaseous neurotransmitter in the central nervous system, diffusing from its cells of origin and affecting surrounding neuronal tissue in evanescent three-dimensional waves. This is also true of the hypothalamus, where amongst other activities NO inhibits stimulation of corticotrophin-releasing hormone (CRH) and vasopressin release by inflammatory stressors, effects thought to be mediated by binding with soluble guanylate cyclase (sGC). Carbon monoxide is being increasingly recognised as another gaseous neuromodulator, but with principal effects on other hemoproteins such as cyclo-oxygenase, and a distinctly different profile of localisation. NO is predominantly a pro-inflammatory agent in the periphery while CO is often anti-inflammatory. In the hypothalamus, the actions of CO are also distinct from those of NO, with marked antagonistic effects on the inflammatory release of vasopressin, both *in vitro* and *in vivo*, but with little involvement in the regulation of CRH. Thus, it would appear that these apparently similar gases exert quite distinct and separate effects, although they cause broadly similar overall changes in the secretion of neuroendocrine stress hormones. We conclude that that these two gases may play significant but different roles in the control of the neuroendocrine stress response, but one common feature may be attenuation of inflammation-induced release of stress hormones.

INTRODUCTION

In a seminal paper by Verma and colleagues (1993), first characterizing carbon monoxide (CO) as a neural messenger in the central nervous system (CNS), it appeared that there may be redundancy with the nitric oxide (NO) pathway: both gases shared a common transduction pathway, i.e. the activation of soluble guanylyl cyclase (sGC) in target cells. However, it was also shown that CO may function in the central nervous system (CNS) in a manner that is independent of NO, for example, in CNS regions where sGC is unassociated with any NO synthase (NOS) activity but is closely related anatomically to the enzyme leading to CO formation, heme oxygenase (HO).

In spite of this, assuming that CO and NO shared a common second messenger and hence broadly acted in a similar manner, most of the studies on CO and

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NO actions, particularly those on long-term potentiation, have been conducted on the underlying assumption that they share a common second messenger and thus act through a common intracellular signalling system (Zhuo *et al.*, 1993; Meffert *et al.*, 1994). In this review, we briefly discuss whether, and to what extent, the above concepts are relevant, or indeed true, in the field of studies concerning the neuroendocrinology of stress.

BIOSYNTHESIS OF NO AND CO

Endogenous NO is a by-product of L-arginine catabolism through the NOS pathway. Under basal conditions, NO is generated by two NOS isoforms, endothelial and neuronal NOS (eNOS and nNOS, respectively); both are calcium-dependent and expressed in a constitutive manner in endothelial cells and neurons, where baseline production of NO is associated with such physiological functions as the control of vascular tone and neuronal plasticity. The third isoform, inducible NOS (iNOS), is not calcium-dependent and is synthesised primarily by cells of the immune-inflammatory lineage such as resident macrophages (microglia in the CNS) and monocytes upon induction by inflammatory mediators, including the cytokines interleukin-1 and –6, TNF-α and by bacterial endotoxins or viruses (Moncada *et al.*, 1991).

So far as CO is concerned, the endogenous synthesis of the gas is associated with heme catabolism. The latter occurs through the hydrolysis of heme groups at the α-methene bridge in heme by HO; this reaction yields equimolar amounts of biliverdin, iron and CO (Tenhunen *et al.*, 1969). Two isoforms of the enzyme have been fully characterised: inducible and constitutive HO (HO-1 and HO-2, respectively) (Maines, 1997). To date, a single report has described a third HO isoform, HO-3 (McCoubrey *et al.*, 1997). Several mammalian tissues and organs, including the brain, have a high capacity to generate CO under basal conditions because of constitutive HO expression. Moreover, the body can rapidly adapt to an increased demand for heme catabolism by over-expressing HO-1; the latter shows limited expression under resting conditions, but is sensitive to a variety of oxidative stressors (e.g. iron and other metals, hemin itself), transcriptional control being under the influence of multiple factors including NF-κB and AP-1 (Dong *et al.*, 2000).

Thus, NO and CO biosynthetic pathways show a similar profile of regulation, with basal production principally influenced by constitutive isoforms whereas several pathological stimuli induce increased synthesis of the gases. However, certain peculiarities of each system require discussion. Firstly, differences exist in the tissue and organ distributions of the constitutive isoforms: in the brain, constitutive HO shows a much greater expression compared to nNOS and eNOS, suggesting that the generation of CO under basal conditions exceeds by far that of NO (Maines, 1997); secondly, stimuli able to induce HO-1 are better described as oxidative stressors, whereas iNOS is exquisitely sensitive to immune-inflammatory challenges which are not necessarily associated with an oxidative burden.

BIOSYNTHESIS OF NO AND CO IN THE HYPOTHALAMUS

On the basis of their gaseous nature, NO and CO appear to exert biological activities in or near their sites of production, acting as autocrine or paracrine regulators. Thus, a preliminary requirement for them to be considered as central regulators of the stress axis is that the gases are synthesised within the hypothalamus itself. Indeed, all of the enzymes leading to the production of both NO and CO have been detected within this region.

Within the hypothalamus, all three NOS isoforms have been identified; eNOS is associated with endothelial cells in the hypothalamic tissue, where it might indirectly influence neuronal function by regulating local blood flow (Ceccatelli *et al.*, 1992), while nNOS is localized to discrete cell clusters and groups. The majority of nNOS mRNA, as revealed by *in situ* hybridization, is located within the paraventricular (PVN) and supraoptic nuclei (SON), with scattered

cells distributed in the anterior periventricular region as well as in the anterior hypothalamus, primarily in the diagonal band of Broca and the islands of Calleja (Bredt *et al.*, 1990; Grossman *et al.*, 1994). The magnocellular neurones of the PVN and SON produce either vasopressin or oxytocin, and project to the posterior pituitary. The parvocellular PVN neurones produce corticotrophin-releasing hormone (CRH) and vasopressin, and project to the median eminence to release the peptides into the hypophysial portal circulation to regulate ACTH secretion. There is evidence favouring co-storage of nNOS and each of these neuropeptides within these neurons (Navarra *et al.*, 2000). NOS co-storage appears to be a widespread phenomenon in the hypothalamus: NOS-like immunoreactivity has been shown to be co-localised with substance P, cholecystokinin, galanin, enkephalin, somatostatin and with vasoactive intestinal polypeptide (Reuss *et al.*, 1995; Yamada *et al.*, 1996). Co-storage of nNOS with several peptides in specific neuronal populations suggests at least one mode of action of NO in these neurons, i.e. as an autocrine agent controlling the release, and perhaps the synthesis, of neuropeptides.

There is little iNOS present in the hypothalamus under basal conditions (Bhat *et al.*, 1996), but after *in vivo* stimulation with endotoxin we and others have recently shown an increase in message transcription throughout the brain, and within the hypothalamus in particular (Wong *et al.*, 1996; Jacobs *et al.*, 1997; Satta *et al.*, 1998). Such an increase is associated with the recruitment of glial cells within the tissue. In this regard, *in vitro* experiments by our group show that purified primary cultures of rat cerebral cortical microglia generate higher amounts of NO compared to astrocytes under resting conditions: moreover, the former are far more responsive to lipopolysaccharide (LPS) challenge, with significant increases in NO production as early as 1 hour after the addition of endotoxin (Figure 1).

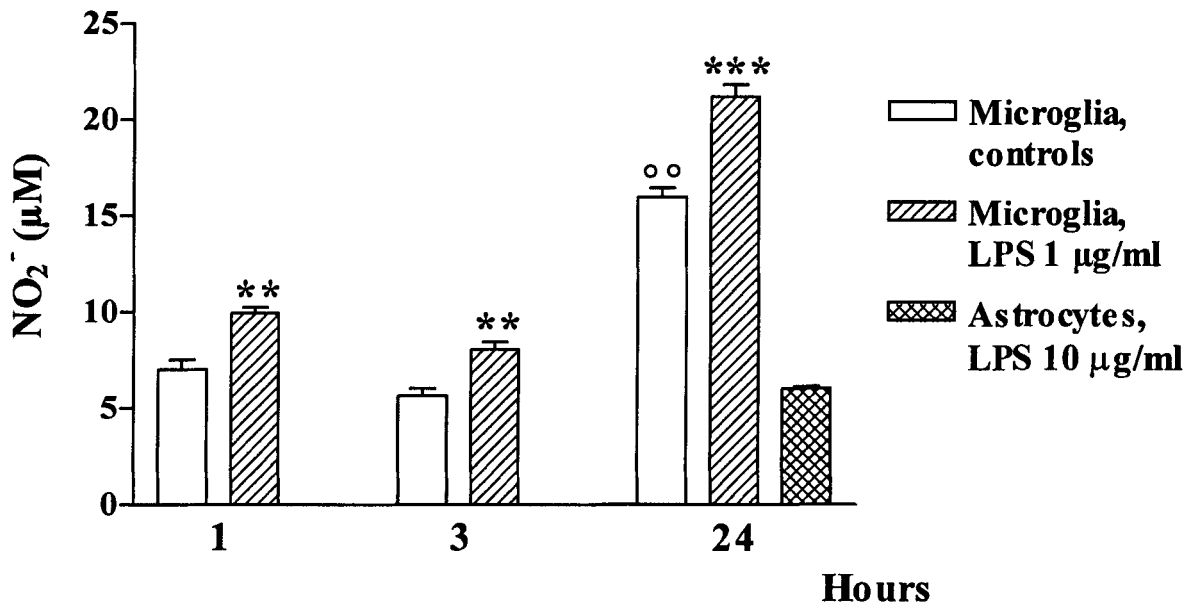

FIGURE 1 Primary cultures of rat cortical microglia ($5x10^4$ cells/cm^2) produce and release NO in a time-dependent manner, with levels after 24 h of incubation being significantly higher than those found after 1 and 3 h; 1 μg/ml of bacterial lipopolysaccharide (LPS) significantly increased NO production with respect to controls at all time-points tested. Conversely, NO production by rat cortical astrocytes was undetectable under resting conditions, and could only be measured after 24 h of incubation in the presence of 10 μg/ml of LPS. NO was measured as nitrite content in the incubation media (500 μl/well). Results are expressed as the means ± standard error of the mean of 4 replicates per group. ** and ***: P<0.01 and P<0.001 versus control microglia respectively; °°: P< 0.01 versus control microglia at 1 and 3 h (assessed by ANOVA followed by *post-hoc* Bonferroni test)

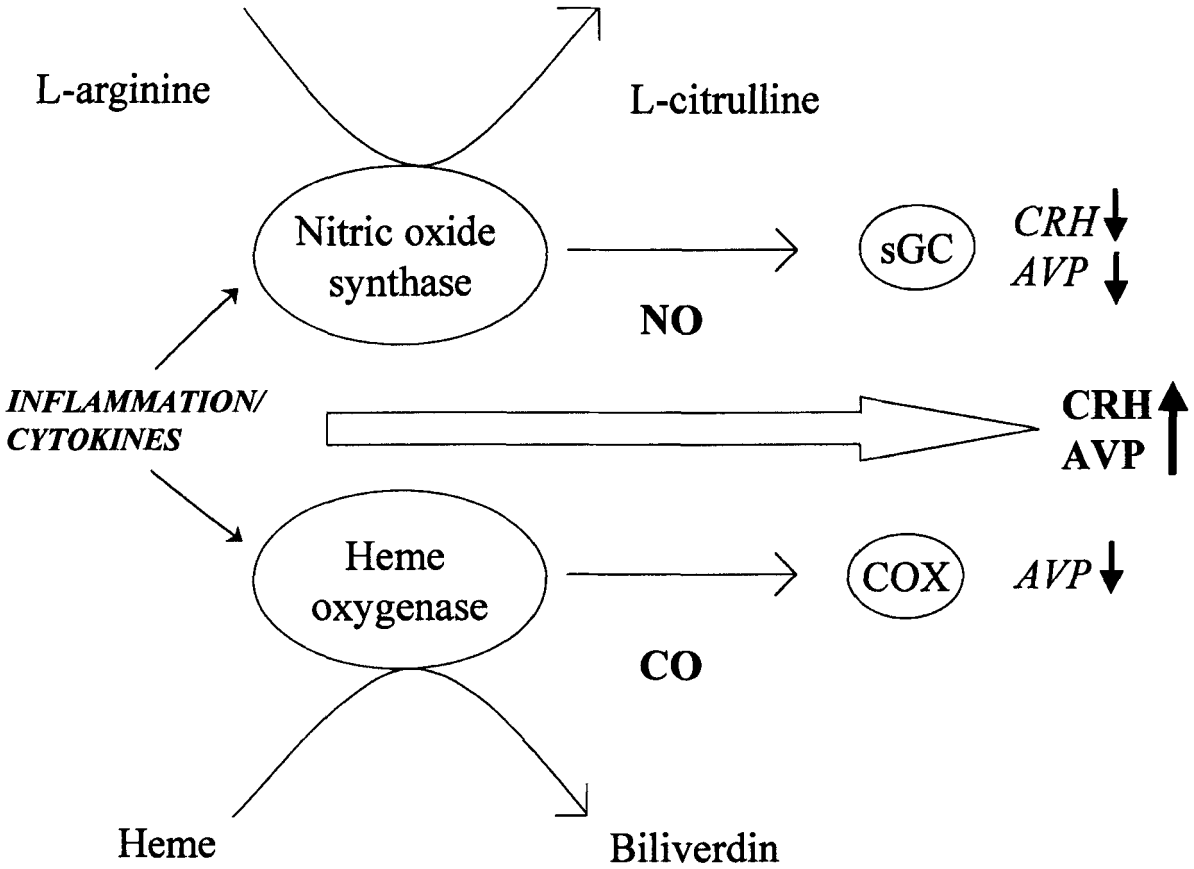

FIGURE 2 Schematic diagram of the involvement of the gaseous neuromodulators nitric oxide (NO) and carbon monoxide (CO) in the neuroendocrine stress response. sGC = soluble guanylate cyclase; COX = cyclo-oxygenase

As far as CO is concerned, most of the relevant information has been obtained by the group of Maines and colleagues (Ewing and Maines, 1992). Under basal conditions, the bulk of CO-forming activity in the hypothalamus is accounted for by HO-2, which is diffusely widespread in this area. Conversely, HO-1 is present in tiny amounts compared to peripheral tissues and organs, and is localised in sparse groups of neurons, including the PVN. Evidence from *in vitro* studies shows that, upon appropriate stimulation, HO-1 can also be expressed by glial cells (Dwyer *et al.*, 1995; Kitamura *et al.*, 1998).

Thus, both CO and NO are generated under basal conditions within the hypothalamus, with the former likely to be produced in far higher amounts. Furthermore, they may both undergo increased biosynthesis as a consequence of chemical stressors. However, specific studies conducted by our group to address this point clearly showed that iNOS, but not HO-1, mRNA expression is increased in the hypothalamus and other rat brain areas after *in vivo* treatments with bacterial LPS (Jacobs *et al.*, 1997; Satta *et al.*, 1998). These findings indicate that, at least as far as endotoxin challenge is concerned, the NO pathway appears to be set to respond to such stimuli with acute, brief and presumably adaptive increases, according to which NO might be considered either as a mediator of stress responses to immune challenges

or, alternatively, as a factor opposing the exaggerated stress activation induced by the endotoxin. Conversely, the CO system better fits with a model of tonic, ie continuing, control over hormonal stress responses.

SIGNALLING PATHWAYS

One obvious way for both NO and CO to exert biological activities is via the interaction with heme groups; as a consequence of this binding, either activation or inhibition of the relevant hemoprotein may occur. The best characterised of these interactions is that with sGC; NO binds heme iron, inducing a conformational change (i.e. the rupture of iron five-coordinated binding with His-105) which causes up to 400-fold activation of the enzyme. CO also binds sGC but its effect is much weaker compared to that of NO, and it is questionable as to whether CO is a physiological activator of sGC (Koestling and Friebe, 2000). Recent studies with the synthetic benzylindazol derivative YC-1 led to the postulation of the existence of an allosteric site on sGC; binding of YC-1 (or a putative endogenous ligand) to this site markedly potentiates the enzymatic activation induced by CO (McLaughlin *et al.*, 2000; Koestlig and Friebe, 2000).

Beside sGC, other hemoproteins have been shown to mediate certain biological effects of NO and CO. Cyclo-oxygenase (COX) is activated by NO to produce prostaglandins (PGs); this occurs via both increased activity as well as over-expression of the inducible COX isoform (Zamora and Billiar, 2000). We have also shown that CO increases PG production and release in the CNS *in vitro* (Mancuso *et al.*, 1997a; Mancuso *et al.*, 1998). Conversely, binding of CO to the heme ring of cytochrome P450, and subsequent enzymatic inhibition, appears to mediate the vasodilatory effects of the gas (Coceani *et al.*, 1996). Furthermore, NOS is itself a hemoprotein, and modulation of NOS activity by CO has been described (Maines, 1997).

A number of biological activities of NO are *not* mediated by the interaction with hemoproteins: these include RNA ribosylation, protein nitrosylation, formation of peroxynitrite ions, and the inhibition of ribonucleotide reductase. Similarly, CO has recently been shown to exert *anti*-inflammatory activities via the activation of a mitogen-activated protein (MAP) kinase pathway, with no apparent involvement of either NO or cGMP; interestingly, NO exerted a potent *pro*-inflammatory activity in this experimental paradigm (Otterbein *et al.*, 2000).

EFFECTS ON THE RELEASE OF STRESS PEPTIDES

Arginine vasopressin and oxytocin

Increases in NOS gene expression and protein biosynthesis in the PVN and SON were observed after dehydration or water deprivation (reviewed by Navarra *et al.*, 2000). The above phenomena suggested a control mechanism according to which stimuli able to increase arginine vasopressin and oxytocin secretion simultaneously activate the generation of NO which, in turn, blunts exaggerated vasopressin and oxytocin secretion. This notion has been reinforced by experiments in which pharmacological manipulation using NO precursors or inhibitors is followed by reduced or enhanced release of vasopressin and oxytocin respectively; as expected, inhibition by NO was especially evident when vasopressin and oxytocin secretion was initially elevated, for example as a result of KCl-induced depolarization *in vitro* (Yasin *et al.*, 1993; Summy-Long *et al.*, 1993).

Some years after, our group undertook a series of *in vitro* experiments looking at the effect of CO on vasopressin and oxytocin release from isolated rat hypothalami. The pharmacological approach was similar to that previously employed in studies of NO, with a HO substrate, hemin, and specific HO inhibitors used to enhance or reduce hypothalamic CO production respectively. We found that KCl-stimulated release of both vasopressin and oxytocin was inhibited in a concentration-dependent manner by hemin, an effect counteracted by the addition of HO inhibitors; the above response was mimicked by CO-enriched incu-

bation media but not by biliverdin, providing clear evidence that CO was mediating the effects of hemin on peptide release (Kostoglou-Athanassiou *et al.*, 1996; Mancuso *et al.*, 1997b).

A preferential pattern of regulation by CO of vasopressin release also emerged from one other *in vitro* paradigm: bacterial LPS exerted a paradoxical inhibitory action on both CRH and vasopressin release from rat hypothalamic explants, an effect apparently mediated by inhibitory pathways involving NO and CO (Navarra, 1995). While the blockade of nitric oxide synthase reversed the minor inhibitory effects of LPS on both CRH and vasopressin release, the HO inhibitors, Zn-protoporphyrin-9 and Sn-mesoporphyrin-9, were able to convert the small degree of inhibition of vasopressin release into marked stimulation, while having no effect on CRH release (Kostoglou-Athanassiou *et al.*, 1998).

Subsequent *in vivo* experiments confirmed that CO exerts an inhibitory action on vasopressin release; this effect was particularly easy to demonstrate under conditions of increased vasopressin secretion induced by LPS. Indeed, pre-treatment with HO inhibitors given by the intracerebroventricular route had no clear effect on basal vasopressin levels but dramatically enhanced the modest increase in peptide release induced by LPS (Mancuso *et al.*, 1999).

Corticotrophin-releasing hormone

A large number of studies have investigated the effects of both NO and CO on CRH release; in spite of these efforts, the issue currently remains controversial, although the prevailing opinion is that the net effect of both NO and CO may vary depending on the stimulus causing the increase in CRH secretion.

Early *in vitro* studies showed stimulation of CRH release by NO (Karanth *et al.*, 1993; Raber and Bloom 1994; Sandi and Guaza, 1995). On the contrary, our group found that increased NO generation was associated with the inhibition of stimulated CRH release from hypothalamic explants (Costa *et al.*, 1993). This finding was consistent with the results of *in vivo* studies by the group of Rivier and colleagues (Rivier and Shen, 1994; Rivier 1995; Turnbull and Rivier, 1996): they showed that the inhibition of NOS further enhanced the activation of the hypothalamo-pituitary-adrenal (HPA) axis caused by endotoxaemia or local inflammation in the rat. While activation of the HPA axis is an integrated phenomenon that only partially depends on hypothalamic CRH, the picture was rendered even more complex by the finding that NO appears to be positively coupled with HPA activation induced by so-called physico-emotional stimuli (Rivier 1998). In another piece of the puzzle, NO appeared to mediate the increase in CRH release induced *in vitro* by the HIV viral coat protein Gp120. Indeed, Gp120-induced CRH release was abolished by NOS inhibitors; we also found that iNOS, but not eNOS or nNOS, was involved, and the gas was able to induce new CRH synthesis after as early as one hour of incubation in the presence of Gp120. The above effects of NO were not mediated by interleukin-1 (Costa *et al.*, 2000; Pozzoli *et al.*, 2000).

Theoretical models have been proposed to explain the above discrepancies (Kim and Rivier, 1998; Navarra *et al.*, 2000); at present, none of these seems to fully explain this complex issue. Most of the differences observed may be well accounted for by differences in the experimental models adopted; thus, any attempt to draw general conclusions from the above experimental findings should be treated with caution. Possibly, a fine-tuned balance involving the precise level of NO produced, its sites of production (PVN versus median eminence), the NOS isoforms involved and the time-course of NO production, might account for the fact that the same molecule plays opposite roles under apparently broadly similar conditions.

Controversy also exists concerning the putative role of CO on CRH release. In experiments on primary cultures of rat hypothalamic cells, Parkes and colleagues (1993) found that hematin, a hemin isoform also hydrolyzed by HO, or CO-enriched incubation media, were able to increase CRH secretion, an effect counteracted by the HO inhibitor Zn-protoporphyrin-9. Almost at the same time, our group found that hemin produces *inhibition* of interleukin-1β- and KCl-stimulated CRH release from rat hypothalamic explants, this effect being antagonised by Zn-pro-

toporphyrin-9 (Pozzoli *et al.*, 1994). However, the effect of hemin on CRH release was weaker compared to that observed on vasopressin and oxytocin release, although the vasopressin and oxytocin release is likely to be from magnocellular neurones rather than parvocellular neurones involved in ACTH regulation

The *in vivo* counterpart of these studies is represented by experiments looking at the effects of CO on the HPA axis, generally assessed through the measurement of circulating ACTH and glucocorticoids in the rat. In the very same experiments showing that inhibition of HO markedly potentiated LPS-induced stimulation of vasopressin release (see above), we observed only a weak effect on corticosterone levels, not significantly higher compared to that of LPS given alone (Mancuso *et al.*, 1999). This finding seems to confirm that, at least as far inflammatory-mediated stress is concerned, CO primarily regulates vasopressin release, while its action on CRH release is negligible. On the contrary, in stress responses induced by such physico-emotional stimuli as foot-shock, CO (in close analogy to NO) would appear to mediate the *increase* in stress hormone levels occurring in this experimental paradigm (Turnbull *et al.*, 1998; Kim and Rivier, 2000).

SUMMARY

Many of the effects of NO and CO on neuroendocrine stress mechanisms appear to show attenuation of inflammatory stress responses, especially NO inhibition of the HPA axis via effects on both CRH and vasopressin release, while CO seems to exert its effects predominantly on vasopressin (Fig. 2). By contrast, both gases may mediate stimulatory responses to other stressors. The discordance between the effects of these two gases, and the possible different second messengers involved, suggests that these gases have complementary rather than strictly summative or redundant roles. At present, clinical correlates of these influences remain a matter of intriguing speculation.

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