Response to Reviewer 1

Comment #1: Although my previous recommendation, the Authors did not detailed the pro-inflammatory role of CO; in lines 59-61 only a brief mention has been done and this is not balanced because, throughout the paper, the Authors continued to magnify the anti-inflammatory effects of CO.

Response: We have added those sentences per your suggestion.

(Lines 69–78) [CO and BR may also play dual roles (i.e., pro-inflammatory and anti-inflammatory) in a variety of organs and tissues [13, 14], depending on their concentration and the signaling pathways involved. Sustained activation of the HO-1-CO pathway may facilitate the development of neuroendocrine disturbances characteristic of age-related neuroinflammatory diseases [15]. To prevent neurotoxicity, BR must be glucuronidated and excreted in the bile. An excessive accumulation of BR in the brain would result in kernicteric damage. Similar to CO and BR, free iron has a pro-oxidant capacity in a redox-active form, leading to lipid, protein, and DNA damage. Overexpression of the HO-1 gene in oxidatively stressed astroglial cells may perpetuate intracellular reactive oxygen species (ROS) generation, oxidative mitochondrial injury, and non-transferrin-derived iron deposition within the mitochondrial compartment [16].]

Comment #2: The Authors must provide details about the ability of CO to blunt the hypothalamus-pituitary-adrenal axis (see previous comments).

Response: We have added details regarding the ability of CO to blunt the hypothalamus-pituitary-adrenal axis.

(Lines 59–68) [On the other side, the HO inhibitor Sn-protoporphyrin-9 has been proven to amplify the significant activation of the hypothalamus-pituitary-adrenal axis induced by bacterial lipopolysaccharide administration in male Wistar rats [11]. This observation indicates a protective role for HO in counteracting potentially dangerous surges of serum vasopressin levels, leading to hypothalamic vasopressin depletion. Accordingly, in in vitro studies, the formation of CO within the hypothalamus has been associated with inhibition of the release of hormones, such as corticotropin-releasing hormone, arginine vasopressin and oxytocin, involved in hypothalamus-pituitary-adrenal axis activation [12]. These findings suggest that the HO-CO pathway may have a neuroendocrine modulatory role, preventing over-exuberant activation of the hypothalamus-pituitary-adrenal axis during stress.]

Comment #3: Furthermore, several mistakes have been spotted among the references. Reference #11 and #12 do not refer directly to CO’s pro-inflammatory activity; the Authors may want to take advantage of reading previous comments and cite proper references.
Response: In accordance to the reviewer’s advice, we have changed the needed References.

(Line 69–70) [CO and BR may also play dual roles (i.e., pro-inflammatory and anti-inflammatory) in a variety of organs and tissues [13, 14], depending on their concentration and the signaling pathways involved.]

Comment #4: The interaction between BR and NO has not been adequately addressed (see previous comments).

Response: We have added the interaction between BR and NO per your suggestion.

(Lines 234–249) [In the absence of exogenous stimuli, BR upregulates the phosphorylation of the cAMP responsive element binding (CREB) factor and the production of NO. The extracellular Ca^{2+} chelator ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) interferes with this pathway [62], suggesting a role for BR in Ca^{2+}-mediated CREB and nNOS activation. Both CREB and NO are considered important factors contributing to synaptic plasticity and memory consolidation by regulating the expression of brain-derived neurotrophic factor (BDNF) [63, 64]. Therefore, BR may boost the repair process countering the deficiency of neurotrophic factors following brain injury. On the other hand, treatment of PC12 cells with BDNF or neurotrophic growth factor increased the signaling to Akt (Protein Kinase B) and extracellular signal-regulated kinases (ERKs), which are crucial factors for survival, and these effects were markedly reduced by BR [62]. Therefore, these observations indicate an important action of BR on survival signaling mediated by neurotrophins, with either inhibitory or agonistic effects based on growth factor availability.
In addition, BR serves as an endogenous scavenger for RNS by denitrosylating the thiol group of proteins and non-protein molecules [65]. In response to exogenous hydrogen peroxide, BR markedly decreases ROS generation in PC12 cells [62].]

Comment #5: I do not understand the reason why the Authors decided to include preclinical data on HO-1 and BVR post-translational modifications in AD the presence of specific data obtained in human samples (brain and serum/plasma): the Authors should take into proper consideration this suggestion.

Response: We have added the data showing the expression of both HO and biliverdin reductase (BVR) in Alzheimer’s disease.

(Lines 325–338) [Recently, a down-regulation of HO-2 and BVR has been demonstrated in post-mortem brain tissues of AD subjects as compared to tissues of age-matched controls. These changes were found in the hippocampus, an area associated with cognitive functions such as learning and memory [82-84]. Moreover, a significant increase in the phosphorylation of HO-1 serine residues was observed in the hippocampus of AD subjects [84]. As a result of phosphorylation, HO-1 can become a target for oxidative posttranslational modifications of the protein structure, with consequent functional impairment. In addition, repression of HO-1 mRNA has been reported under strong and
sustained pro-oxidant conditions such as hypoxia, heat shock, or interferon-γ [85-87]. Treatment of HUVECs with the iron chelator, desferrioxamine, or hypoxia reduced HO-1 mRNA expression [87], possibly preventing toxic accumulation of HO metabolites such as iron and CO. Though HO-1 mRNA expression in plasma has been reported to be markedly reduced in AD subjects as compared to healthy controls [88], recent studies show enhanced HO-1 protein levels in plasma, hippocampus, and cerebellum of AD subjects [84, 89]. Therefore, the reports of HO-1 levels in AD are still controversial.]

Comment #6: Reference #76 refers to a wide review on the neuroprotective effects of statins; once again, my suggestion is to provide references of original articles dealing with this issue (see previous recommendations).

Response: We have added references to the original experiments.

(Lines 345–351) [Administration of the cholesterol-lowering agent atorvastatin can induce BVRA and HO-1 protein expression in the parietal cortex of aging dogs [91-93], resulting in increased BR levels. In addition, atorvastatin lowered oxidative/nitrosative stress biomarkers, such as 3-nitrotyrosine, in the same cortical area [92, 93], leading to the reduction of oxidative stress-mediated neuroinflammation. Size discrimination learning error scores negatively correlated with BVRA protein levels and BVR activity [92]. Therefore, atorvastatin-mediated HO-1 and BVR upregulation may be associated with reduced oxidative stress and improved cognitive functions in the aging brain.]

Comment #7: Finally, as recommended also by Reviewer #2, the repression of HO-1 gene must be deeply addressed (see previous comments).

Response: We have added the repression of HO-1 gene under oxidative stress conditions.

(Lines 331–334) [In addition, repression of HO-1 mRNA has been reported under strong and sustained pro-oxidant conditions such as hypoxia, heat shock, or interferon-γ [15-17]. Treatment of HUVECs with the iron chelator, desferrioxamine, or hypoxia reduced HO-1 mRNA expression [17], possibly avoiding toxic accumulation of HO metabolites such as iron and CO.]