Response to Comments by Reviewer 1

Comment #1: Heme oxygenase (HO) exists in two main isoforms, namely HO-1 and HO-2; HO-3 is considered a spliced-variant of HO-2 and does not have any enzymatic activity. HO-2 is constitutive (and not constant, see line 34) and serves as a sensor not only for oxygen, but also for other gaseous molecules.

Response: Thank you for your comment. We have changed those sentences per your suggestion.

(Lines 34–37) [HO-2, a constitutive isoform of HO, is present in high levels in the liver, brain, and testes. In the brain, HO-2 functions as a sensor for oxygen as well as other gaseous molecules and regulates vascular function. HO-3 is a pseudogene found in the rat brain and does not have enzymatic activity.]

Comment #2: In addition, HO and the by-product carbon monoxide (CO), have been demonstrated, several years ago, to behave as pro-inflammatory molecules, since they blunt the adrenal response to stressors (Mancuso et al., J Neuroimmunol 1999; Mancuso et al., Neuroimmunomodulation 1997). In addition, and in support of the pro-inflammatory nature of HO, is the evidence that this latter stimulates prostaglandin synthesis in the rat brain (Mancuso, Pistritto et al., Mol Brain Res 1997; Mancuso et al., J Neurosci Res 2006). Several papers have also shown as HO-1 undergoes repression under strong and long-lasting pro-oxidant conditions in order to avoid toxic accumulation of both CO and ferrous iron (Palozza et al., Antioxid Redox Signal 2006; Shibahara et al., Exp. Biol Med 2003; Nakayama et al., BBRC 2000).

Response: We have added the effects of HO metabolites on the pro-inflammatory response.

(Lines 55–61) [However, HO and its metabolites are Janus-faced. In this regard, HO-1 in astrocytes of the aging and diseased central nervous system (CNS) can be an effector of deleterious stimuli, leading to neuronal injury [1]. Administration of the HO inducer hemin can increase pro-inflammatory prostaglandin E2 levels in rat hypothalamic explants and in primary cultures of rat hypothalamic astrocytes [2]. CO and BR may also have dual roles (i.e., pro-inflammatory and anti-inflammatory) in several different organs and tissues [3, 4], depending on the concentration and the signaling pathway involved.]
In reactive glia, astrocyte-specific overexpression of HO-1 in conditions of oxidative stress leads to neuronal injury by releasing neurotoxic molecules such as IL-1β and TNFα [1, 5]. In transgenic mice overexpressing human HO-1 in their astrocytes, HO metabolites promote mitochondrial sequestration of non-transferrin iron, oxidative stress-mediated substrate modification within the mitochondria, and subsequent mitophagy [5]. Co-culture of PC12 cells with HO-1 overexpressing astrocytes induced PC12 cell death, which was reduced by treatment with deferoxamine, an iron chelating agent [6]. Therefore, the neurotoxic role of HO-1 in oxidative stress-conditioned astrocytes may stem from excessive iron deposition.]

[However, sustained upregulation of HO and its metabolites in the brain may exacerbate neural injury. Therefore, the disparate properties of HO and its metabolites under various neuropathological conditions must be carefully considered for clinical approaches.]

Comment #3: A similar dual role, i.e. neuroprotective and neurotoxic, has been demonstrated for bilirubin (BR) (Mancuso, Neuropharmacology 2017). The Authors did not mention the link between BR and nitric oxide, quite important as a determinant of neuroinflammation (Mancuso et al., Neurosci Lett 2012; Mancuso et al., J Neurosci Res 2008; Mancuso et al., Antioxid Redox Signal 2006). Because of BR-NO interaction, the formation of N-nitro-BR has been also shown (Barone et al., J Cell Mol Med 2009). Since herpesviruses infection has been proposed as a potential mechanism underlying neuroinflammation, noteworthy is the study by Santangelo et al. (Front Pharmacol 2012) dealing with the antiviral activity of BR.

Response: In accordance with your comment, we have added the link between BR and nitric oxide to the text.

The link between BR and NO has been reported as a determinant of neuroinflammation [3]. BR upregulates the nNOS/NO axis in primary rat cerebellar granule neurons exposed to serum starvation or conditions of neurotrophin deficiency [3]. In addition, BR serves as an endogenous scavenger for RNS by denitrosylating the thiol
group of proteins and non-protein molecules [7]. BR can be formed by the HO-BVR pathway, and HO is co-expressed with BVR in the brain [8]. Therefore, BR may reduce peroxynitrite production when the HO-BVR pathway is activated in the TBI brain. One possible target that may lead to activation of the HO-1-BVR pathway in TBI might be CO.]

**Comment #4:** The Authors did not mention at all the role played by both HO and biliverdin reductase (BVR) in Alzheimer’s disease (Barone et al., Free Rad Biol Med 2012; Barone et al., Biochim Biophys Acta 2011; Barone et al., J Alzheimers Dis 2011) as well as the druggability of both HO and BVR as targets of atorvastatin (Barone et al., J Neurochem 2012; Barone et al., Int J Neuropsychopharmacol 2012).

**Response:** We have added the role played by both HO and biliverdin reductase (BVR) in Alzheimer’s disease.

(Lines 285–304) [2.2.4. AD

In patients with AD, failure of recent memory and other intellectual functions is observed. Amyloid precursor protein (APP) generates the β-amyloid (Aβ) peptide, postulated to participate in the neurotoxicity found in AD. Neuronal loss and reactive astrocytes may be associated with Aβ-peptide toxicity and the deposition of neurofibrillary tangles containing hyperphosphorylated tau in AD [1]. HO-2 interacts with APP, and APP inhibits HO activity [9]. Treatment of rat hippocampal neurons with CORM-2 protects them against Aβ-induced toxicity [10]. A marked reduction in neuronal BR levels may be related to an increased sensitivity to H$_2$O$_2$-induced neurotoxicity in transgenic APPswe mice [9], suggesting that HO metabolites, such as CO and BR, may play cytoprotective roles in AD. Similar to TBI, pericyte loss is also promoted in AD, consequently leading to the accumulation of neurotoxic Aβ in transgenic APPswe mice [11]. The link between HO metabolites and functional pericyte recovery is of great interest in AD.

BVRA can be modified by oxidative/nitrosative stress into tyrosine nitration (3-NT-BVRA), which is increased in the hippocampus of 12 and 18 month-old 3xTg-AD mice compared with wild-type mice [12]. The 3xTg-AD mice have three mutant human genes,
i.e., APP<sup>swe</sup>, PS1<sub>M146V</sub>, and tau<sub>P301L</sub>, and show reductions in BVRA protein levels at 3, 6, and 12 months old [12]. Impaired BVRA activity, partly via 3-NT-BVRA, and reduction in BVRA expression may result in increased oxidative stress and inflammation in AD [12, 13]. Administration of the cholesterol lowering agent atorvastatin can induce BVRA and HO-1 protein levels [14], resulting in increases in CO and BR, consequently reducing oxidative stress-mediated neuroinflammation.

References


