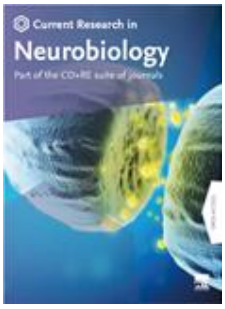
Peer Review Overview



Manuscript Title: The SARS-CoV-2 spike glycoprotein interacts with MAO-B and impairs mitochondrial energetics

Received	Mar 14, 2023
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1st Revision Submitted	Aug 21, 2023
Accepted	Sep 25, 2023

1st Decision letter

Reference: CRNEUR-D-23-00022
Title: The SARS-CoV-2 spike glycoprotein interacts with MAO-B and impairs mitochondrial energetics
Journal: Current Research in Neurobiology

Dear Mary Ellen,

I am so sorry for the extreme delays we have faced procuring reviewers. I was ready to do something drastic, proceeding with only one review, but we were fortunate a second reviewer could complete in record time.

Please see below the reviewer comments. They recommend minor and major revision accordingly.

I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Aug 29, 2023.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

Current Research in Neurobiology values your contribution and I look forward to receiving your revised manuscript.

Kind regards,

Christopher I. Petkov
Editor in Chief
Current Research in Neurobiology

Comments from Editors and Reviewers:

Reviewer #1:

The present work by Cuperlovic-Culf, Harper and co-workers appears as a continuation of a very recent work by some of the co-authors of this paper (Cuperlovic-Culf, Harper et al. Sci Rep 2021, 11, 1-14), which established a link between modified brain MAO B activity following the SARS-CoV-2 infection based on the interaction between MAO B and the spike protein from the virus. The performed work is comprehensive, nicely planned and well executed, and involves a large number of biological and biochemical experiments with a consistent conclusion that that the MAO B···spike protein interaction alters the MAO B activity and leads to changed concentrations of PEA and PEA-related metabolites. The latter led authors to suggest that the demonstrated SARS-CoV-2 impact on the monoaminergic pathway is responsible for an increased incidence of neurological disturbances in patients infected with the SARS-CoV-2 virus.

However, the present paper seems to contradict some of the conclusions from the mentioned previous work, which needs to be mentioned and discussed.

Firstly, in their earlier paper, the authors reported the PEA concentration increase in studied patients which would imply a reduced MAO B activity in SARS-CoV-2 infected individuals, while the present analysis suggests decreased PEA concentrations and an increased MAO B activity. This needs to be discussed and clarified.

Secondly, in their earlier paper, the authors reported computational modeling results that seem to show that the interaction between the spike protein and MAO-B may alter neurotransmitter metabolism by affecting the access of neurotransmitters to the MAO-B active binding site. Yet, if that would be the case, it would hinder the approach of PEA to the active site and rule out the possibility of an increased PEA metabolic conversion that the authors are reporting here. The mentioned computational results were questioned by the paper quoted here (Hok et al. Comput Struct Biotechnol J 2022, 20, 1254-1263) where this disturbance in the MAO B activity was ascribed to the changed electrostatic environment in the MAO B active site following the interaction with the spike protein, which, in the light of the present results, appears to be more correct. This also needs to be discussed in the present work.

Also, the quoted work by Hok et al. clearly demonstrated that the effect of the MAO B···spike protein interactions on the MAO B metabolic activity is highly dependent on (i) the SARS-CoV-2 strain, and (ii) the physiological substrate inspected, which should be mentioned here. In this context, regarding the first aspect, the authors should state which SARS-CoV-2 strain was used throughout their work, while the second aspect should be mentioned by hinting that some other substrates could likely reveal different trends within the same experimental setup. The latter would agree, for example, with the work by B. Shen et al., Cell 2020, 182, 59, where a hindered MAO activity and lower concentrations of serotonin-related metabolites were experimentally demonstrated in the sera of COVID-19 infected patients.

In conclusion, it is my belief that the present work by Cuperlovic-Culf, Harper and co-workers provides new and important insight into COVID-19 mediated brain disturbances and is publishable. Yet, the work must undergo a major revision considering the mentioned concerns before it is further considered.

Reviewer #2:

This is a very well-done study. The rationale behind the experiments were clearly described and each set of experiments was guided by clear and concise hypotheses. The authors provide considerable evidence that, at least in their experimental system, that the S protein and MOA-B interact. They go one to show that this interaction results in an increase in MOA-B activity. The evidence in support of these first two findings was quite compelling, as each point was supported by complimentary methods. The authors then go on to link the increased MOA-B activity to altered cellular redox homeostasis, as well as an increased susceptibility to cell death in response to MPTP+ exposure. Given the rigor of the study in its present form, the conclusions made by the authors align with the evidence presented. The main limitation of the study is that the majority of experiments were done in a single cell line. This is not meant to be a criticism, as the cell biology in the paper was very well done; however, the authors should consider adding a section in the discussion that lays out the limitations of their work, particularly with respect to extrapolating their findings to SARS-CoV-2 infection, as well as Parkinson's disease. In addition, below is a list of suggested revisions that the authors might consider adding to the final published work:

* Some of the language in the paper seems overly subjective. For examples, on line 115, the authors state "Here we substantially advance....." Given the limitations of the study, perhaps it would be better to be more objection here and just state "Here we demonstrate that....."
* The authors present LDH activity as evidence in support of a shift in oxidative glycolytic metabolism. Such claims would be better supported using more rigorous assays, such as quantification of lactate excretion or metabolomics.
* To support the claim of redox alterations induced by S protein-MAO-B interaction, could the authors consider measuring GSSG in the culture media. Presumable, the H2O2 being produced by MOA-B is being buffered by the GSH pool and the reason that GSSG is not increasing in the cell is likely because GSSG is exported.
* For the H2O2 emission experiments in Figure 4, it is surprising that Antimycin A addition did not increase emission beyond that of succinate? Could the authors please address this. Also, did the assay include a condition prior to the addition of substrates? If so, can this be added to the figure?
* In the MPTP+ experiments, could the authors show that the inhibitor of MOA-B activity rescues the MPTP+ induced death in S expressing cells.

1st Author Response Letter

Response to comments from Editors and Reviewers:

Comments from Reviewer 1:

The present work by Cuperlovic-Culf, Harper and co-workers appears as a continuation of a very recent work by some of the co-authors of this paper (Cuperlovic-Culf, Harper et al. Sci Rep 2021, 11, 1-14), which established a link between modified brain MAO B activity following the SARS-CoV-2 infection based on the interaction between MAO B and the spike protein from the virus. The performed work is comprehensive, nicely planned and well executed, and involves a large number of biological and biochemical experiments with a consistent conclusion that that the MAO B···spike protein interaction alters the MAO B activity and leads to changed concentrations of PEA and PEA-related metabolites. The latter led authors to suggest that the demonstrated SARS-CoV-2 impact on the monoaminergic pathway

is responsible for an increased incidence of neurological disturbances in patients infected with the SARS-CoV-2 virus. However, the present paper seems to contradict some of the conclusions from the mentioned previous work, which needs to be mentioned and discussed.

We thank the reviewer for their valuable feedback and recommendations. Below, please find detailed responses to the specific concerns and suggestions raised by the reviewer.

1.Firstly, in their earlier paper, the authors reported the PEA concentration increase in studied patients which would imply a reduced MAO B activity in SARS-CoV-2 infected individuals, while the present analysis suggests decreased PEA concentrations and an increased MAO B activity. This needs to be discussed and clarified.

We thank the reviewer for this feedback and the opportunity to clarify this important point.

Our 2021 *Scientific Reports* study (1) included two separate datasets from previously published cohorts. The first dataset was from a case–control delirium cohort of post-operative patients undergoing knee and hip replacements (2,3). The second data set was a cohort of control patients or patients with COVID-19 disease (4).These two studies are summarized below:

The metabolomics analyses from the case–control postoperative delirium cohort were performed in an observational cohort study using blood and CSF samples of preoperative patients aged over 65 years who were delirium-prone or not (control) and were undergoing hip and knee replacements. Importantly neither group had COVID-19. In this study (1), the control subjects had a higher concentration of phenethylamine (PEA) in CSF and a lower concertation of PEA in the blood. In contrast, the subjects that were prone to delirium had a lower concentration of phenethylamine (PEA) in CSF and a higher concertation of PEA in the blood. While PEA concentration was slightly reduced in in the delirium group in both the CSF and blood, it did not reach statistical significance ($p = 0.1$).

The analyses from the control and COVID-19 patients were done on data collected by Shen *et al.* (2020) (4), and quantitative metabolomic and proteomic profiling was performed in serum samples. Importantly, PEA was not quantified in this study. Instead, analyses of the data obtained by Shen *et al* (4) revealed that the metabolites that differed between severe and mild cases of COVID-19 could be related to pathways involving monoamine oxidases (1). Specifically, the ceramide/sphingosine 1-phosphate ratio has been shown to regulate the activity of monoamine oxidases (5), where increases in the Ceramide/S1P Ratio and inhibition of SphK1 were shown to increase the activity of MAO-A in cardiomyocytes. In the data obtained from Shen *et al.* (4), Cuperlovic-Culf *et al.* (1) calculated the ceramide/sphingosine 1-phosphate ratios for both the control and COVID-19 patient samples (Shen *et al.,* 2020), and showed that the ratio was higher in patients with mild COVID-19 compared to controls, and highest in patients with severe COVID-19 disease (Figure 5c in (1)). Therefore, the increase in the ceramide/sphingosine 1-phosphate ratios observed in the patients with COVID-19 disease suggests an increase in MAO activity. Moreover, as demonstrated in the Shen *et al*. (2020) paper, the levels of serotonin decrease as the severity of COVID-19 increases, supporting the idea that the activity of MAO is higher with increasing severity of COVID-19 disease (4).

We agree with the reviewer that this needs to be clarified in the manuscript, and have edited the discussion. Line 559: “Increased metabolism of other monoamine substrates has also been observed. Specifically, levels of serotonin were found to be lowest in serum from patients with severe COVID-19, consistent with the possibility that the activity of MAOs may increase with increasing severity of COVID-19 disease (4,6).”

2.Secondly, in their earlier paper, the authors reported computational modeling results that seem to show that the interaction between the spike protein and MAO-B may alter neurotransmitter metabolism by affecting the access of neurotransmitters to the MAO-B active binding site. Yet, if that would be the case, it would hinder the approach of PEA to the active site and rule out the possibility of an increased PEA metabolic conversion that the authors are reporting here. The mentioned computational results were questioned by the paper quoted here (Hok et al. Comput Struct Biotechnol J 2022, 20, 1254-1263) where this disturbance in the MAO B activity was ascribed to the changed electrostatic environment in the MAO B active site following the interaction with the spike protein, which, in the light of the present results, appears to be more correct. This also needs to be discussed in the present work.

The computational analysis in our previous paper showed that the docking site for the spike protein is likely the membrane mediated substrate entrance to the active site of MAOB (1). However, this analysis did not consider the regions of MAO-B bound to the mitochondrial membrane. The subsequent analysis by Hok *et al.* did incorporate the membrane dynamics of MAO-B in their analysis which disagreed with the theory that the spike protein was blocking the entrance of substrates to MAO-B (7). Instead, they theorized that the spike protein was modifying the electrostatic environment and affinity of substrate binding. Specifically, Hok *et al.* show that the interaction between MAO-B and the spike protein can decrease the substrate cavity particularly with the South African variant B.1.351. However, they note that this interaction with the South African variant B.1.351 is very stable and actually increases the affinity of MAO-B for its substrates. Hok *et al.* highlight that this aligns with findings of decreased serotonin in patients with severe COVID-19 disease (4).

To clarify these findings in the context of our work, we have incorporated the following text into the introduction and discussion sections:

Line 99: Computational modelling suggests that the interaction between the S protein and MAO-B may alter neurotransmitter metabolism by affecting access of neurotransmitters to the active binding site of MAO-B, or by modifying the electrostatic environment (1,8).

Line 543: Initially the SARS-COV-2 spike protein was thought to interfere with the substrate entrance to the MAO-B active site (1). However, additional modelling by Hok *et al.* suggests that the SARS-COV-2 spike protein modifies the electrostatic environment of the substrate binding site for MAO-B (Hok et al., 2022). Moreover, mutant SARS-CoV-2 variants have different ACE2 binding affinities and different SARS-CoV-2 strains may have varying degrees of activation or inhibition of MAO-B activity, suggesting that the resulting effects on MAO-B may depend on the surrounding environment such as proximity to lipid membranes and certain spike variants. Therefore, future studies should experimentally determine MAO-B substrate binding affinity when complexed with various spike protein variants, both biochemically and in the context of living systems.

3.Also, the quoted work by Hok et al. clearly demonstrated that the effect of the MAO B···spike protein interactions on the MAO B metabolic activity is highly dependent on (i) the SARS-CoV-2 strain, and (ii) the physiological substrate inspected, which should be mentioned here. In this context, regarding the first aspect, the authors should state which SARS-CoV-2 strain was used throughout their work, while the second aspect should be mentioned by hinting that some other substrates could likely reveal different trends within the same experimental setup. The latter would agree, for example, with the work by B. Shen et al., Cell 2020, 182, 59, where a hindered MAO activity and lower concentrations of serotonin-related metabolites were experimentally demonstrated in the sera of COVID-19 infected patients.

The reviewer raises an important point. We selected 2-phenylethylamine as the substrate for MAO-B, as it shows the highest specificity towards MAO-B compared to MAO-A in human cerebral cortex (MAO-A Km=140 ± 22, MAO-B Km=4 ± 2)(9), but agree that it would be important to explore the effects of the SARS CoV-2 spike protein on MAO-B activity using different substrates. Importantly, serotonin was quantified in the serum metabolomics in the 2020 *Cell* paper (Shen et al., 2020), which showed that serotonin decreased in patients with COVID-19 disease as the severity of COVID-19 increased, suggesting MAO activity is increased in the patients with COVID-19 disease.

We have incorporated this limitation in the discussion and have also added information about the strain and the importance of comparing different SARS CoV-2 strains in the manuscript:

Line 563: Importantly, since our analysis was limited to SH-SY5Y neuroblastoma cells with PEA as a substrate, the increase in MAO-B activity should be verified in other brain cell types and with other monoamine substrates.

Line 543: Initially the SARS-COV-2 spike protein was thought to interfere with the substrate entrance to the MAO-B active site (1). However, additional modelling by Hok *et al.* suggests that the SARS-COV-2 spike protein modifies the electrostatic environment of the substrate binding site for MAO-B (Hok et al., 2022). Moreover, mutant SARS-CoV-2 variants have different ACE2 binding affinities and different SARS-CoV-2 strains may have varying degrees of activation or inhibition of MAO-B activity, suggesting that the resulting effects on MAO-B may depend on the surrounding environment such as proximity to lipid membranes and certain spike variants. Therefore, future studies should experimentally determine MAO-B substrate binding affinity when complexed with various spike protein variants, both biochemically and in the context of living systems.

Comments from Reviewer 2:

This is a very well-done study. The rationale behind the experiments were clearly described and each set of experiments was guided by clear and concise hypotheses. The authors provide considerable evidence that, at least in their experimental system, that the S protein and MOA-B interact. They go one to show that this interaction results in an increase in MOA-B activity. The evidence in support of these first two findings was quite compelling, as each point was supported by complimentary methods. The authors then go on to link the increased MOA-B activity to altered cellular redox homeostasis, as well as an increased susceptibility to cell death in response to MPTP+ exposure. Given the rigor of the

study in its present form, the conclusions made by the authors align with the evidence presented. The main limitation of the study is that the majority of experiments were done in a single cell line. This is not meant to be a criticism, as the cell biology in the paper was very well done; however, the authors should consider adding a section in the discussion that lays out the limitations of their work, particularly with respect to extrapolating their findings to SARS-CoV-2 infection, as well as Parkinson's disease. In addition, below is a list of suggested revisions that the authors might consider adding to the final published work.

We thank the reviewer for their valuable feedback and recommendations. We agree with the reviewer that a limitation of the study is that most of the experiments were only performed in SH-SY5Y cells, and have incorporated this into the discussion (Line 563: Importantly, since our analysis was limited to SH-SY5Y neuroblastoma cells with PEA as a substrate, the increase in MAO-B activity should be verified in other brain cell types and with other monoamine substrates.)

Below, please find detailed responses to the suggestions raised by the reviewer.

1.Some of the language in the paper seems overly subjective. For examples, on line 115, the authors state "Here we substantially advance....." Given the limitations of the study, perhaps it would be better to be more objection here and just state "Here we demonstrate that....."

We agree with the reviewer, and have removed the subjective wording as per the reviewer's suggestion. Line 115: Here we demonstrate that the SARS-CoV-2 S protein interacts with cellular MAO-B in vitro and augments MAO-B activity.

2.The authors present LDH activity as evidence in support of a shift in oxidative glycolytic metabolism. Such claims would be better supported using more rigorous assays, such as quantification of lactate excretion or metabolomics.

We thank the reviewer for this excellent suggestion. We analyzed extracellular lactate levels in the culture medium, which were greater for the SH-Spike cells. We have incorporated these findings in the manuscript and in Figure 3g.

Line 418: As a proxy measure of anaerobic metabolism, we next quantified LDH activity and extracellular lactate concentrations. Consistent with a shift in oxidative to glycolytic metabolism, SH-SY5Y-Spike cells demonstrated increased LDH activity and higher lactate concentrations in culture medium compared to SH-EV cells (Figure 3f and 3g).

Line 626: The drastic reduction of p53 in cells expressing the S protein may contribute to impaired mitophagy and elicit the Warburg effect by decreasing parkin expression (10), which is supported by our findings of increased LDH activity and extracellular lactate.

3.To support the claim of redox alterations induced by S protein-MAO-B interaction, could the authors consider measuring GSSG in the culture media. Presumable, the H2O2 being produced by MOA-B is being buffered by the GSH pool and the reason that GSSG is not increasing in the cell is likely because GSSG is exported.

The reviewer raises an interesting point. In our study, the SH-Spike cells exhibited decreased protein expression of glutathione peroxidase 4 (GPX4), and lower intracellular concentrations of both reduced glutathione (GSH) and total glutathione (GSH + 2GSSG). In contrast, oxidized glutathione (GSSG) and the ratio of GSH:GSSG were not different compared to SH-EV cells, despite evidence of increased ROS production.

Decreases in intracellular glutathione can result from 1) a decrease in glutathione synthesis, 2) an increase plasma membrane glutathione efflux transport, 3) glutathione oxidation and adduct formation, or 4) dysregulated GSSG secretion from the ER (11). These processes are challenging to accurately quantify as efflux transporters have low catalytic efficiency, there are many unstable different forms of thiols/adducts/conjugates that are present in low concentrations, and there are multiple pathways of glutathione (re)synthesis. The intracellular concentrations of glutathione the cell range between 1–10 mM depending on the cell type (12), of which 80-90% exists in its reduced state. Whereas extracellular glutathione was found to be in the micromolar range in plasma, cerebral spinal fluid, and rat brain cortex (13).

In some neural cells, the multidrug resistance-associated protein 1 (MRP1) can export GSH, GSSG, and glutathione S-conjugates (14,15). However, the extracellular release of GSSG from neural cells has only been observed during oxidative stress conditions (15). Due to the low micromolar concentrations of extracellular glutathione, we are unable to quantify both GSH and GSSG in cell media. Furthermore, it is likely a combination of the mechanisms stated above that contribute to the observed decreases in glutathione in SH-Spike cells. In support of this, there is evidence that SARS-CoV-2 decreases concentrations of cellular thiols by decreasing *de novo* glutathione biosynthesis due to low cellular availability of cysteine, and increases in glutathione efflux via increases in MRP1 protein expression (16).

Because quantification of extracellular GSSG is challenging to quantify, we have instead incorporated this as a discussion point in Line 587: The observed decreases in glutathione in SH-Spike cells in our study could be a result of decreased glutathione synthesis, formation of various forms of thiol conjugates, and/or increased glutathione efflux via the multidrug resistance-associated protein 1 (MRP1) (13,15). In support of this, depletion of cellular thiols in SARS-CoV-2-infected Vero-E6 cells has been attributed to both decreased *de novo* glutathione biosynthesis due to low availability of cysteine, and also increased glutathione efflux via increased MRP1 protein expression (16).

4.For the H2O2 emission experiments in Figure 4, it is surprising that Antimycin A addition did not increase emission beyond that of succinate? Could the authors please address this. Also, did the assay include a condition prior to the addition of substrates? If so, can this be added to the figure?

The finding that H_2O_2 rates were highest after succinate is due to reverse electron transport. In our protocol, succinate is added in the presence of glutamate and malate, but the ADP is absent (state 4). Under these conditions, electrons are supplied to the CoQ pool while protonmotive force is high. Since ADP is absent, reverse electron transport is elicited, and electrons are transported in reverse from CoQH2 to complex I to reduce NAD+ to NADH, which generates large amounts of ROS (17–20). However, the

addition of ADP that follows succinate in our protocol dissipates protonmotive force, and the high rates of ROS generated from reverse electron transport ceases. Thus, under these conditions, the inhibition of complex III with antimycin A blocks reverse electron transfer, leading to decreased ROS generation (21).

No other conditions were measured prior to the addition of the substrate. Only the necessary reagents required to detect H_2O_2 were added prior to the addition of substrates. These included Amplex red and horseradish peroxidase to detect H_2O_2 through the formation of resorufin, and digitonin to permeabilize the cell membrane.

5.In the MPTP+ experiments, could the authors show that the inhibitor of MOA-B activity rescues the MPTP+ induced death in S expressing cells.

We thank the reviewer for this excellent suggestion. In response, we conducted $MPTP^+$ experiments with rasagiline and used trypan blue exclusion. We found that rasagiline partially reversed $MPTP^+$-induced cell death, and have incorporated these findings in the manuscript and Figure 6a.

Line 497: Following 24 hours of treatment, trypan blue exclusion tests revealed that $MPTP^+$ elicited cell death in SH-Spike that was partially reversed with rasagiline (Figure 6a).

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Accept Letter

Dear Professor Harper,

Thank you for submitting your manuscript to Current Research in Neurobiology.

I am pleased to inform you that your manuscript has been accepted for publication.

My comments, and any reviewer comments, are below.

Your accepted manuscript will now be transferred to our production department. We will create a proof which you will be asked to check, and you will also be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

We appreciate and value your contribution to Current Research in Neurobiology. We regularly invite authors of recently published manuscript to participate in the peer review process. If you were not already part of the journal's reviewer pool, you have now been added to it. We look forward to your continued participation in our journal, and we hope you will consider us again for future submissions.

CRNEUR aims to be a unique, community-led journal, as highlighted in the Editorial Introduction. As part of this vision, we will be regularly seeking input from the scientific community and encourage you and your co-authors to take the survey.

We would also like to invite you to take part in our CRNEUR Author Question & Answer (Q&A), which could get published alongside your article and help to promote it. We suspect you might have an interesting story of perseverance or team work that was required for the research study to complete, or a diversity of perspectives that you might share, as a way of inspiring others about neuroscience.

Kind regards,

Christopher I. Petkov
Editor in Chief
Current Research in Neurobiology

Editor and Reviewer comments:

Reviewer 1: The revised version of the work by Cuperlovic-Culf, Harper and co-workers represents a much improved contribution. The authors addressed all of my concerns and suggestions in appropriate ways. The manuscript now reads well and is much clearer. I consider it as an important contribution to the field of molecular neurobiology and COVID-19 induced neurological disturbances, which warrants publication in the CRN journal. I have no further comments to make, therefore I can recommend the revised version of this work for publication in its current form.

Reviewer 2: The authors have done an excellent job of addressing all points raised during the initial review.

-------- End of Review Comments --------