progress in neurobiology

Peer Review Overview

Manuscript Title: "The Intracellular Renin-Angiotensin System: Friend or Foe. Some Light from the Dopaminergic Neurons"

Received	28-Feb-2020
1st Decision	12-Jun-2020
1st Revision Submitted	18-Jun-2020
2nd Decision	18-Jul-2020
2nd Revision Submitted	20-Aug-2020
Accepted	04-Oct-2020

1st Decision Letter

Dear Prof Labandeira-Garca,

Thank you for submitting your manuscript to Progress in Neurobiology. We have received comments from reviewers on your manuscript. Your paper should become acceptable for publication pending suitable minor revision and modification of the article in light of the appended reviewer comments.

When resubmitting your manuscript, please carefully consider all issues mentioned in the reviewers' comments, outline every change made point by point, and provide suitable rebuttals for any comments not addressed.

Please resubmit your manuscript by Aug 11, 2020.

We look forward to receiving your revised manuscript.

Kind regards,

Kimberley Raab-Graham
Associate Editor

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Comments from the Editors and Reviewers:

Reviewer 1

The authors are experts in the RAS, and the present review focuses on its interactions with the dopamine system in the substantia nigra and presents a very detailed and complete literature on the intracellular RAS with a heavy emphasis on their own published literature.

First, the authors succinctly describe the basis of the RAS, and the principal direct and additional components as proposed by the literature. This is followed by the analysis of the literature on the relationship between the brain RAS and the brain dopamine system, not only in the brain and the substantia nigra, but in peripheral organs as well.

The review continues with a more detailed analysis of the literature on the reported intracellular RAS, with focus on the mitochondrial RAS and the functional analysis of mitochondrial Angiotensin receptors, and the reported impact of the mitochondrial RAS on aging and disease.

The authors conclude that the intracellular RAS regulates multiple physiological functions, and that the close relationship between the paracrine and circulating RAS with the intracellular RAS contributes to the effects of RAS overactivity on the development of multiple disorders in peripheral organs and in the brain.

The review is very well written and much of the literature has been included and placed within the general context of the manuscript.

The major limitation of the review is that the literature has been taken at face value. There is no critical analysis of the literature and no consideration of the literature pitfalls related to two important methodological errors. One, experiments when conclusions are drawn from experiments when the physiological to pathological levels of compounds and RAS factors are unknown or orders of magnitude lower than those obtained after administration of the factors or compounds in question. The normal or the pathological levels of compounds must match the amounts administered to elicit a response. For example, what are the normal/pathological levels of brain Angiotensin II (and Ang (1-7)) in the substantia nigra?

The second main limitation repeated in the literature is the continuous use of commercially available non-selective antibodies that have been proven to originate erroneous results. For these reasons the review may be strengthen by the addition of a paragraph pointing out that although highly attractive and organized around the hypothesis proposed, a significant part of the conclusions from the literature may be erroneous and in need to reassessment. This unfortunate lack of antibody characterization, for example for AT1, AT2 and Mas receptors extends to many other GCPRs and a significant number of publications have not taken this limitation in consideration. By pointing these limitations, the authors may warn the readers that perhaps not all that is published is necessarily correct.

Minor points:

- The authors may wish to modify their reference to the AT4 receptors, that are not, as the authors point out, specific. Cloning of the receptors revealed that they correspond to an insulin-regulated membrane aminopeptidase depending peptidase DOI: 10.1007/s00018-004-4246-1.
- "Norepinephrin" should be "norepinephrine" and “dopamine b-hydroxylase" should be "dopamine β-hydroxylase".

Reviewer 2

The review present a very interesting topic is well written with good bibliographic support. The authors have been working for many years in the subject and they have several publications that are included in the manuscript together with other author's findings going in the same direction. The authors might know that there are many controversies regarding brain angiotensin system, respect synthesis, receptors localization, precursors ... could the authors refer to this last respect? Considering the authors are expert in brain Ang II, could

they include some aspects regarding Ang II in the glia? This last is suggested in relation with neuroinflammation, the complexity of brain pathologies, and considering that Ang II and AT1-R are present in glial cells.

1st Author Response Letter

Reviewer 1

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First, the authors succinctly describe the basis of the RAS, and the principal direct and additional components as proposed by the literature. This is followed by the analysis of the literature on the relationship between the brain RAS and the brain dopamine system, not only in the brain and the substantia nigra, but in peripheral organs as well.

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The review is very well written and much of the literature has been included and placed within the general context of the manuscript.

Response: Thank you very much for your positive comments.

The major limitation of the review is that the literature has been taken at face value. There is no critical analysis of the literature and no consideration of the literature pitfalls related to two important methodological errors. One, experiments when conclusions are drawn from experiments when the physiological to pathological levels of compounds and RAS factors are unknown or orders of magnitude lower than those obtained after administration of the factors or compounds in question. The normal or the pathological levels of compounds must match the amounts administered to elicit a response. For example, what are the normal/pathological levels of brain Angiotensin II (and Ang (1-7)) in the substantia nigra?

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Response: I agree that in this field (and practically any other research field) not all that is published is necessary correct. However, note that most of the conclusions and comments

of our review are based on several studies or studies confirming results with several methodological approaches. I also agree that the use of non-physiological doses in experimental treatments and the use of commercial antibodies without confirmation of specificity and/or confirmation of results using simultaneous methodology (RT-PCR, binding experiments, functional studies with agonists/antagonists) may generate confusing results and conclusions.

Following the reviewer´s suggestion, we have included a paragraph on these problems in the revised version of the manuscript (page 5 last paragraph- page 6 first paragraph).

Regarding the specific question/example, we have determined nigral levels of AngII (or Ang1-7) in control animals, models of parkinsonism or aged animals in several previous studies. We usually separate different angiotensin peptides in the tissue sample using HPLC and quantify pure samples of the different peptides using the corresponding EIA kit (RIA in early studies). Levels of Ang II in the SN were similar to those found by other authors such as Gao et al 2017, DOI: 10.18632/oncotarget.15732 (around 30pg/mg that increased to 60 pg/mg after treatment with rotenone). Our values for Ang 1-7 in the SN of young rats were 19.17± 1.6 pg/mg protein (with about 50% decrease in aged rats).

Minor points:

- *The authors may wish to modify their reference to the AT4 receptors, that are not, as the authors point out, specific. Cloning of the receptors revealed that they correspond to an insulin- regulated membrane aminopeptidase depending peptidase DOI: 10.1007/s00018-004-4246-1.*
- *In addition, "norepinephrin" should be "norepinephrine"; "dopamine b-hydroxylase" should be "dopamine β-hydroxylase".*

Response:

- We agree with the reviewer about AT4 receptors. As we focused on intracellular RAS and AT4 was just mentioned in the introduction, we did not go into this controversy. However, we have clarified this point and included the suggested reference in the revised version of the manuscript (page 5, second paragraph).
- The spelling errors have been corrected.

Reviewer 2

The review presents a very interesting topic is well written with good bibliographic support. The authors have been working for many years in the subject and they have several publications that are included in the manuscript together with other author's findings going in the same direction.

Response: Thank you very much for your positive comments.

The authors might know that there are many controversies regarding brain angiotensin system, respect synthesis, receptors localization, precursors ... could the authors refer to this last respect?

Response: This suggestion is very close to that commented by Reviewer #1. The use of inadequate methodology or single methodological approaches has led to controversial results as indicated by the reviewer. Even some authors (....) have questioned the existence

of a brain RAS because they were unable to detect renin in the brain, and suggested that Ang II is uptaken from the blood. Other authors have measured low levels of renin, and more importantly, there are high levels of pro-renin and pro-renin receptors in the brain that have catalitic activity similar to renin. In addition, different RAS components and the corresponding mRNA have been shown in cultures of neurons and glial cells (i.e. in the absence of a blood source).

In response to the reviewer´s suggestion we now included a two new paragraphs on this question (page 5 last paragraph- page 6 first paragraph; Page 7 first paragraph) and 7 additional references were included.

Considering the authors are expert in brain Ang II, could they include some aspects regarding Ang II in the glia? This last is suggested in relation with neuroinflammation, the complexity of brain pathologies, and considering that Ang II and AT1-R are present in glial cells.

Response: I agree with the reviewer that the effect of brain RAS on regulation of neuroinflammation, particularly the microglial response, is a crucial aspect of the brain RAS and its role in brain diseases. We have investigated on this question in a considerable number of studies. However, we have focused on this question in other recent review, and this is the reason why we did not go into details in the present review on the intracellular RAS.

Following the reviewer´s suggestion we mentioned this point (Page 7 second paragraph and cited several (4) recent studies from our lab and others in the revised version of the manuscript.

Editor

This review was written before the Covid-19 crisis (pre-submission-inquiry on February 3), and as I suggested in a previous e-mail to the Editor (May 20), the inclusion of at least a paragraph on the possible role of the intracellular RAS, particularly intracellular ACE2, in the effects of Sarcov-2 on cells appears now necessary. We have included this paragraph in the revised version (Section 4.4: page 15-17) and 7 new references.

2nd Decision Letter

Dear Prof Labandeira-Garca,

Thank you for submitting your manuscript to Progress in Neurobiology.

We have completed our evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. We invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Sep 16, 2020.

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.

Please make sure that you involve a native English speaker to proof read your manuscript.

Progress in Neurobiology values your contribution and we look forward to receiving your revised manuscript.

Kind regards,

Kimberley Raab-Graham
Associate Editor

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Editor and Reviewer comments:

Reviewer 2

The authors have improved the manuscript and I consider that the manuscript in the present form offers a more complete vision of brain Ang II system.

Reviewer 3

The revised review by Labandeira-Garcia and colleagues has reviewed the current literature including their own studies on the functional role of the intracellular renin-angiotensin system (IRAS). The review focuses on the neuronal RAS with possible interactions with the dopaminergic system, although peripheral IRAS.in the nucleus and mitochondria are discussed as well. Moreover, the classical (ACE-Ang II-AT1R) and alternative (ACE2-Ang-1-7-MasR) arms of the IRAS are reviewed, particularly their potential interaction to regulate cell activity. The main concerns from the previous reviewers were the non-specificity of the tools or approaches to characterize components of the IRAS, as well as strength of evidence for intracellular generation of Ang II and Ang-(1-7). Overall, the authors have addressed these concerns in this comprehensive paper that partially succeeds in synthesizing results from numerous studies on an obviously very complex field and propose a reasonable hypothesis on the functional aspects of the intracellular system. This reviewer has several comments and suggestions for the revised manuscript as detailed below, in addition to .the need for careful editing.

1. Pg 4. In regards to renin, the authors should cite the original work - Tigerstedt R, Bergman PG: Niere und kreislauf. Scand Arch Physiol 8: 223, 1898
2. Pg 4. The statement that 75% of cardiac angiotensin is paracrine Ang II is confusing. The DeMello and Froehlich review actually state that 75% of cardiac Ang II is from local synthesis. Renin and Aogen are derived from the circulation to participate in local cardiac synthesis. Perhaps the authors should distinguish uptake of RAS components that lead to Ang II or Ang-(1-7) synthesis, local synthesis of RAS components that generate Ang peptides and receptor mediated uptake of Ang II or Ang-(1-7).
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9. Pg 5. What is Angiotensin A? Does this bind to MrgD or AT1R?
10. Pg 5. The authors include Ang III/Ang IV/Ang IV receptor together and Ang III/Ang IV/Ang-(3-4) together. Why? Does Ang III bind to IRAP? What dose Ang-(3-4) bind to and function?
11. Intro (pg 6). The author should cite the paper by Chappell MC Am J Physiol 2016 for a comprehensive discussion of issues with measurement of various components of the RAS.
12. Pg 6. The authors suggest that studies of the RAS are circumspect due to non-physiological concentrations of peptides applied. So, what are the correct concentrations to use in cells or specifically in isolated subcellular components that address this issue?
13. Pg 6. The other concern raised by the authors is the non-specificity of antibodies to the RAS, particularly the Ang receptors - AT1, AT2, Mas, and MrgD. From their published studies, the authors widely utilize these receptor antibodies. Thus, they should comment how they assess specificity and the antibodies they consider specific that would enhance the rigor of their studies.
14. Pg 7. The primary components of the RAS are renin and Aogen which are secreted extracellularly so ifs difficult to envision a local or intracellular synthesis in neurons as represented in their figure.
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28. Pg 16. How do Ang receptors signal on the mitochondria - is there evidence that the requisite G-proteins associated with these receptors are localized to the mitochondria?
29. Pg 17. In regards to Sars-COV-2, the viral complex internalizes with plasma membrane ACE2 and its unclear how this would impact mitochondrial ACE2?
30. Pg 18. Again, can the authors define what are physiological concentrations within the cell for Ang II and Ang-(1-7), as well as a pathological concentration range?
31. Pg 22. Please add the Pendergrass citation (Pendergrass KD et al. BBRC 2009 384(2): 149-154) that first demonstrated that Ang II stimulated ROS in renal isolated nuclei likely through NOX4.

2nd Author Response Letter

Reviewer 2

The authors have improved the manuscript and I consider that the manuscript in the present form offers a more complete vision of brain Ang II system.

Response: Thank you for your comments.

Reviewer 3

The revised review by Labandeira-Garcia and colleagues has reviewed the current literature including their own studies on the functional role of the intracellular renin-angiotensin system (IRAS). The review focuses on the neuronal RAS with possible interactions with the dopaminergic system, although peripheral IRAS.in the nucleus and mitochondria are discussed as well. Moreover, the classical (ACE-Ang II-AT1R) and alternative (ACE2-Ang-1-7-MasR) arms of the IRAS are reviewed, particularly their potential interaction to regulate cell activity. The main concerns from the previous reviewers were the non-specificity of the tools or approaches to characterize components of the IRAS, as well as strength of evidence for intracellular generation of Ang II and Ang-(1-7). Overall, the authors have addressed these concerns in this comprehensive paper that partially succeeds in synthesizing results from numerous studies on an obviously very complex field and propose a reasonable hypothesis on the functional aspects of the intracellular system. This reviewer has several comments and suggestions for the revised manuscript as detailed below, in addition to .the need for careful editing.

Response: Thank you for your comments.

1. *Pg 4. In regards to renin, the authors should cite the original work - Tigerstedt R, Bergman PG: Niere und kreislauf. Scand Arch Physiol 8: 223, 1898*

Response: The reference has been included (page 4).

2. *Pg 4. The statement that 75% of cardiac angiotensin is paracrine Ang II is confusing. The DeMello and Froehlich review actually state that 75% of cardiac Ang II is from local synthesis. Renin and Aogen are derived from the circulation to participate in local cardiac synthesis. Perhaps the authors should distinguish uptake of RAS components that lead to Ang II or Ang-(1-7) synthesis, local synthesis of RAS components that generate Ang peptides and receptor mediated uptake of Ang II or Ang-(1-7).*

Response: We have rewritten the paragraph (page 4) to clarify that in peripheral tissues components derived from circulation can participate in tissue angiotensin synthesis as suggested by the reviewer. In addition we have cited the original paper from Danser et al (1994) from which DeMello and Froehlich obtained the above mentioned data (75%), so that the reader can have more detailed information. Note, however, that this is a very preliminary paragraph and we cannot mention specific RAS components that are introduced in the next paragraph.

3. *Pg 4. Should read as Ang II acts on two G protein-coupled receptors…"*

Response: The typing error has been corrected (page 4).

4. *Pg 5. Should read as "…pro-oxidative and pro-inflammatory effects of AT1 receptor activity…"*

Response: We usually use that expression. However we have changed the sentence following the reviewer´s suggestion (page 5).

5. *Pg 5. I would not characterize IRAP as an Ang IV receptor at all. IRAP is a peptidase and Ang IV may inhibit its activity to stimulate cell signaling, but calling it a receptor connotes a peptide GPRC which it is clearly not..*

Response: The sentence has been rewritten following the reviewer´s suggestion (page 5).

6. *Pg 5. The MrgD does not counteract Ang II by stimulating the Mas receptor. Define almandine as Asp1-Ang-(1-7), a decarboxylated form of Ang-(1-7) at aspartic acid.*

Response: I apologize about MrgD typing error. The word has now been moved to the right place at the end of the sentence (page 5).

Alamandine has been defined as requested (page 5).

7. *Pg 5. Should read as "Altogether, the data suggests a pro-oxidative and pro-inflammatory arm of the RAS that includes…."*

Response: The sentence has been changed as suggested (page 5).

8. *Pg 5. The authors include Ang III as part of the anti-inflammatory axis of the RAS; however, Lloren-Cortes and Harding/Wright groups suggest that Ang III stimulates AT1 receptors and is the ligand for brain AT1R which is clearly different from that in the kidney.*

Response: The reviewer is right that the role of several peptides such as Ang III is controversial and possibly tissue-dependent. This is the reason why we inserted the word “possibly” in the original version of the manuscript. This is brief and general introductory paragraph on RAS before we focus on intracellular RAS, and we think that it should not be

extended going into details on controversies. We have rewritten the paragraph and cited several papers that go into details on this question (page 5-6).

9. *Pg 5. What is Angiotensin A? Does this bind to MrgD or AT1R?*

Response: It is usually considered that Ang A is generated from Ang II, and can bind to AT1R, but also generates alamandine that can bind to MrgD. This is reviewed in detail by Hrenak et al. (2016) as indicated in the revised version of the manuscript. We have rewritten the paragraph considering the reviewer´s comment (page 5-6) (Please, see above point 8).

10. *Pg 5. The authors include Ang III/Ang IV/Ang IV receptor together and Ang III/Ang IV/Ang-(3-4) together. Why? Does Ang III bind to IRAP? What dose Ang-(3-4) bind to and function?*

Response: We have rewritten the paragraph considering the reviewer's comment (page 5-6). Please, see above, point 8.

11. *Intro (pg 6). The author should cite the paper by Chappell MC Am J Physiol 2016 for a comprehensive discussion of issues with measurement of various components of the RAS.*

Response: Several interesting papers from Chappell`s group had been cited in the original version. We have now added this important paper as suggested by the reviewer (page 6).

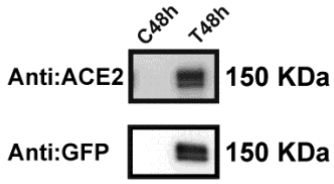
12. *Pg 6. The authors suggest that studies of the RAS are circumspect due to non-physiological concentrations of peptides applied. So, what are the correct concentrations to use in cells or specifically in isolated subcellular components that address this issue?*

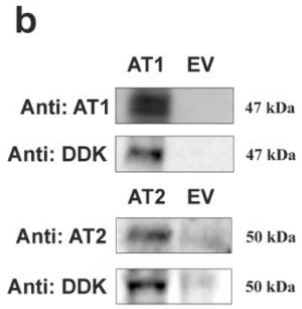
Response: We had to include this statement (and that corresponding to point 13) upon request of a previous reviewer. In the light of the reviewer#3´s comment we have rewritten the sentence. In addition, we have included the reference to Chappell (2016) for concentrations in tissues, cells and subcellular (see also point 30) components (page 6).

13. *Pg 6. The other concern raised by the authors is the non-specificity of antibodies to the RAS, particularly the Ang receptors - AT1, AT2, Mas, and MrgD. From their published studies, the authors widely utilize these receptor antibodies. Thus, they should comment how they assess specificity and the antibodies they consider specific that would enhance the rigor of their studies.*

Response: Following the reviewer´s suggestion, we stated in the manuscript (page 6) that we normally use parallel methods in addition to immunohistochemistry/WB. Furthermore, we confirm specificity in our tissue and species (usually mouse/rat substantia nigra and striatum) with several methods including preadsortion with the corresponding synthetic peptide antigen if available. We have KO mice for different RAS components, but it is known that knockdown of the functional domain does not ensure that the remaining protein will not be recognized by the antibody (which is a usual source of criticisms on lack of specificity of antibodies). The method that we prefer and have reported in several recent papers is the use of western blot analysis of lysates from HEK293 cells transfected with the corresponding GPCR tagged to fusion tail DDK or GFP. The specificiy of the antibodies is confirmed by the presence of a predominant immunoreactive band in positively transfected lysates and the absence of this band in negative controls, which consist of lysates transfected with empty vectors. Then, we order all necessary antibodies from the same reference and batch. Below, we show several examples of characterization of specificity of antibodies:

a. Characterization of ACE2 antibody (c, control; T transfected). Unpublished.





b. AT1 and AT2 antibodies. From Valenzuela et al. (2016) (EV: empty vector/control).

14. *Pg 7. The primary components of the RAS are renin and Aogen which are secreted extracellularly so its difficult to envision a local or intracellular synthesis in neurons as represented in their figure.*

Response: As stated by the reviewer, renin is classically known as a secretory glycoprotein produced, stored and released by the kidney. However, whereas the kidney expresses the transcripts encoding secretory renin, other tissues and cells (including neurons) of rodents and primates additionally or exclusively express transcripts encoding cytosolic renin protein that cannot be secreted, and can act on intracellular angiotensinogen. Several studies have shown that cytosolic renin exerts effects different from and even opposite to those of circulating renin, as cytosolic renin appears to be cell protective (Wanka et al., 2018; Nakagawa et al., 2020). Cyto-renin is also localized within mitochondria, which has been related to its protective effects (Wanka et al., 2018, 2020). Import of cytosolic angiotensinogen into the mitochondria has been also shown (Wilson et al., 2017). This is consistent with our observations of cell protective effects of the intracellular RAS (see below).

In the light of the reviewer´s comment, we have mentioned this point in the revised version (pages 6-7 and 12-13).

15. *Pg 7. The authors should clarify that prorenin bound to the prorenin receptor has catalytic properties similar to active renin.*

Response: The sentence has been changed as suggested and is now more correct (page 8).

16. *Pg 8. Sentence on various effects of dopamine requires a citation*.

Response: We had included several citations at the end of the paragraph. Now, citations were moved to the end of the sentence mentioned by the reviewer (page 8).

17. Pg 8. Authors should define what is an "abnormal interaction" of AT1R and D2R that leads to hypertension

Response: The sentence has been rewritten and clarified (page 9).

18. Pg 9. What other intracellular GPCRs in particular are the authors referring to here?

Response: Several intracellular GPCRs are now mentioned as requested (page 10).

19. Pg 9. Intracellular application of Ang II studies requires a citation.

Response: A couple of citations have been included (page 10).

20. Pg 10. Read as more complicated?

Response: This has been corrected (page 11).

21. Pg 12. Clarify membrane receptors to mean plasma membrane receptors as presumably the mitochondrial receptors are also membrane receptors.

Response: This has been revised and corrected throughout the manuscript.

22. Pg 13. Citations required for 1st paragraph on this page rewarding mitochondrial RAS.

Response: Additional citations have been included as suggested by the reviewer (page 12-13).

23. Pg 14. Clarify exactly what increased ROS "…affects the cytoplasm to induce cell dysfunction"?

Response: Intracellular superoxide (O2-) is primarily produced by the oxidation of NADPH by NAPH oxidase enzymes (NOXs) or by electron leak from aerobic respiration in mitochondria. Superoxide is rapidly converted into hydrogen peroxide (H2O2) by compartment-specific superoxide dismutases (SODs). H2O2 is capable of oxidizing cysteine residues on proteins to initiate redox processes. Alternatively, H2O2 may be converted to H2O by cellular antioxidant proteins, such as peroxiredoxins (PRx), glutathione peroxidase (GPx), and catalase (CAT). When cell antioxidant mechanisms are insufficient and H2O2 levels increase uncontrollably, hydroxyl radicals (OH·) form via reactions with metal cations (Fe^{2+}) and irreversibly damage cellular macromolecules (see for review Schieber and Chandel, 2014)

This has now been explained in the manuscript as requested by the reviewer (page 16).

24. Pg 15. The Kuba reference is for the original SARS virus, not SARS-COV2. Please correct.

Response: The sentence has been corrected (page 18, first line).

25. Pg 15. ACE2 is protective as it degrades Ang II and generates Ang-(1-7). This is key to the overall protective effect of ACE2

Response: The sentence has been rewritten as suggested by the reviewer (page 17, last lines).

26. *Pg 16. Rewrite 1 st sentence of 2nd paragraph. Authors confirmed ACE2 and Ang-(1-7) in primate mitochondria, not mitochondria in brain.*

Response: We isolated mitochondria from samples of brains of monkeys. We reworded the sentence to clarify this (page 18).

27. *Pg 16. Can the authors clarify the Mito localization of significant concentrations of Ang-(1-7), and how would the peptide would access receptors on the mitochondria?*

Response: As indicated above, mitochondrial angiotensins may be imported from the cytoplasm. It is known that the mitochondrial outer membrane contains a multisubunit complex responsible for the recognition and translocation of proteins within mitochondria (Model et al., 2002). However, the possible uptake of different RAS components has not been clarified at the present time. Interestingly, it has been shown that mitochondrial angiotensinogen can be imported from the cytoplasm in kidney cells (Wilson et al., 2017), and that cyto-renin is also imported by the mitochondria (Clausmeyer et al., 1999; Wanka et al., 2018, 2020; Nakagawa et al., 2020; see page 7 and 13). Altogether suggests that angiotensins, including Ang 1-7 may be also produced within the mitochondria. Consistent with this we have observed high levels of ACE2 in the mitochondria. Possible traslocation of angiotensin-receptor complexes to the mitochondria has been commented in page 13.

We have included this information in the revised manuscript (pages 6-7 and 12-13).

28. *Pg 16. How do Ang receptors signal on the mitochondria - is there evidence that the requisite G-proteins associated with these receptors are localized to the mitochondria?*

Response: We now have mentioned the presence of G-proteins in the mitochondria, and included a couple of references (Lyssand and Bajjalieh, 2007; Suofu et al., 2017) (page 13).

29. *Pg 17. In regards to Sars-COV-2, the viral complex internalizes with plasma membrane ACE2 and its unclear how this would impact mitochondrial ACE2?*

Response: Several proteins generated from the SARS-CoV viral genome have mitochondrial targeting sequence (Singh et al., 2020; Yuan et al., 2020). Coronavirus spike proteins contain endoplasmic reticulum retrieval signals that can retrieve spike proteins to the endoplasmic reticulum (Sadasivan et al., 2017; Lontok et al., 2004). Although direct interaction between viral spike protein and mitochondria has not been demonstrated at the present time, the interaction with mitochondrial ACE2 may be via MAMs (mitochondrial associated membrane compartment) (Williamson and Colberg-Poley, 2009), or mechanisms that remain to be clarified. Considering the reviewer´s comment, we have included this explanation in the revised manuscript (page 18-19).

30. Pg 18. *Again, can the authors define what are physiological concentrations within the cell for Ang II and Ang-(1-7), as well as a pathological concentration range?*

Response: Physiological intracellular levels of angiotensins are more difficult to estimate than tissue or circulating levels, as they have been usually estimated in cultured cells, which are affected by the culture conditions and the absence of the regulatory effect of the extracellular RAS and other possible physiological regulatory factors. The levels of intracellular angiotensins varied depending on different cell types and experimental

conditions. However , they were around of 150-200 fmol/mg protein for Ang II and 250-400 fmol/mg protein for Ang 1-7, which may increase and decrease 3-5 times, respectively, under pathological conditions such as high glucose conditions (Lavrentyev et al., 2007; Alzayadneh and Chappell, 2014; see Chappell, 2016 for a detailed review).

We have included this information in the revised version of the manuscript (page 21).

31. Pg 22. Please add the Pendergrass citation (Pendergrass KD et al. BBRC 2009 384(2): 149-154) that first demonstrated that Ang II stimulated ROS in renal isolated nuclei likely through NOX4.

Response: This citation has been added (page 25).