progress in neurobiology

Peer Review Overview

Manuscript Title: "Nilotinib restores memory function by preventing dopaminergic neuron degeneration in a mouse model of Alzheimer's Disease"

Received	24-Jun-2020
1st Decision	10-Aug-2020
1st Revision Submitted	24-Nov-2020
2nd Decision	10-Feb-2021
2nd Revision Submitted	15-Feb-2021
Accepted	28-Feb-2021

1st Decision Letter

Dear Prof. D'Amelio,

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 Oct 09, 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.

To submit your revised manuscript, please log in as an author at https://www.editorialmanager.com/proneu/, and navigate to the "Submissions Needing Revision" folder.

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

Kind regards,

Jeannie Chin
Associate Editor

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Editor and Reviewer comments:

Reviewer #1: In this interesting paper, the authors show electrophysiological and cellular morphology alterations in the lateral part of the VTA in old Tg2576 mice with AD pathology. Changes in VTA would be related to increased autophagosomes and an AD-related kinase, the c-Abl, in older but not younger Tg2576 mice. These modifications could be involved in the reduced hippocampal DA release that has been implicated in AD pathogenesis. Furthermore, they showed that after chronic administration of Nilotinib, a c-Abl inhibitor drug, it prevented alterations of DA neurons in VTA and reversed cognitive dysfunction in older Tg2576 mice.

Overall, this work is well-conceived, the experiments are well designed, and provide more information on the critical role of dopamine activity in AD pathogenesis. Although I think this study deserves to be

considered suitable for publication, I have a couple of comments that could improve the paper.

In the paper, however, the authors neither discussed nor showed how Aβ accumulation or intracellular neurofibrillary tangles has anything to do with VTA cell alterations. It is well known that Aβ accumulation begins in the forebrain and not in the midbrain. The building up of Aβ near VTA will appear at very advanced ages of mice in various transgenic models of AD.

The extracellular accumulation of Aβ in Tg mouse models or direct injections of Aβ into the forebrain areas has been shown to reduce TH terminals that would be involved in retrograde VTA cell abnormalities and even cell death in the VTA. I think the authors should discuss these possibilities.

The reduced activity of another prominent catecholaminergic structure in the early stages of AD is the locus coeruleus. It would be possible that this structure had cellular alterations similar to those of the VTA. Furthermore, damage or photoinhibition of LC has recently been shown to be related to DA hippocampal-dependent cognitive dysfunctions. It would be essential to see in the article a discussion about these possibilities.

Reviewer #2: This is a very interesting manuscript showing that Nilotinib, a c-Abl inhibitor, may protect VTA neurons in Tg2576 mouse model and attenuate cognitive deficit. The study is a reasonable extension of prior studies from this group that show loss early loss of VTA dopaminergic neurons contribute to deficits in Tg2576 model of amyloid pathology. In the current manucript, significant aspect of the manuscript is focused on additional description of the dopaminergic deficits in the Tg2576 mice. While these are all very interesting data, it does not contribute sufficiently to understanding the role of c-Abl in neurodegeneration or AD pathogenesis. Overall, the authors need to provide additional experiments regarding the specificity and the mechanisms of c-Abl and nilotinib. Following specific issues, if addressed will improve the manuscript.

1. Figures 1, 2, and 3 are very nice description of the e-phys deficits of VTA DA neurons. However, the results are only peripherally relevant to the title of the manuscript. Thus, this data should be minimized and summerized into one figure.

2. Figure 4 is also an important data but it is more of an extension of prior study and not directly related to how c-Abl might be acting to cause loss of DA neurons.

3. Authors need to provide summery of actual number and statistical parameters associated with data in Figure 5 (B, C, D).

4. I am not convinced that the effects of nilotinib is directly on the VTA neurons. First, authors need to show what is the status of c-Abl and autophagy in other brain regions, such as hippocampus, cortex, and amygdala. Further, authors need to determine whether nilotinib is impacting APP and Abeta levels in multiple brain regions. Without these data, there is no evidence that nilotinib is acting directly on dopaminergic neurons. Perhaps the authors could also provide immunolocalization of c-Abl activation in VTA neurons (e.g. Karim et al., Mol Neurodegen. 2020).

5. Authors should also provide evidence that nilotinib treatment results in inhibition of c-Abl and alleviated the autophagy deficits seen in Figure 5.

6. The text needs to be revised as many statements are too wordy and with non-standard use of terms.

1st Author Response Letter

We extend our sincere thanks to the Editor and the Reviewers for giving us an opportunity to revise our manuscript in order to strengthen it.

We have dealt with all the points mentioned by the reviewers, and also highlighted all the changes in the text. We provide a point-by-point reply to all the suggestions:

Reviewer #1:

In this interesting paper, the authors show electrophysiological and cellular morphology alterations in the lateral part of the VTA in old Tg2576 mice with AD pathology. Changes in VTA would be related to increased autophagosomes and an AD-related kinase, the c-Abl, in older but not younger Tg2576 mice. These modifications could be involved in the reduced hippocampal DA release that has been implicated in AD pathogenesis. Furthermore, they showed that after chronic administration of Nilotinib, a c-Abl inhibitor drug, it prevented alterations of DA neurons in VTA and reversed cognitive dysfunction in older Tg2576 mice. Overall, this work is well-conceived, the experiments are well designed, and provide more information on the critical role of dopamine activity in AD pathogenesis. Although I think this study deserves to be considered suitable for publication, I have a couple of comments that could improve the paper.

1. In the paper, however, the authors neither discussed nor showed how Aβ accumulation or intracellular neurofibrillary tangles has anything to do with VTA cell alterations. It is well known that Aβ accumulation begins in the forebrain and not in the midbrain. The building up of Aβ near VTA will appear at very advanced ages of mice in various transgenic models of AD.

We thank the Reviewer for the encouraging comments and suggestions. We had previously demonstrated that the selective loss of VTA DAergic neurons, starting at 3-months in Tg mice, occurs at hippocampal pre-plaque stages. Indeed, as the Reviewer suggests, Aβ accumulation is more pronounced in the forebrain rather than in the midbrain. In line with this, we already demonstrated that in 6-month-old Tg256 mice the diffuse staining of APP in the VTA appears to be different from the more intense and focal staining in the hippocampus (Nobili et al., 2017). However, the midbrain dopaminergic neurons express the APPswe construct, thus it is plausible to assume that these cells are more susceptible to Aβ soluble forms than neurons from other brain regions, and susceptibility starts very early, at 3 months of age.

In the revised manuscript now, we provide additional data to demonstrate that VTA dopaminergic neurons of 3-month-old Tg2576 mice show selective autophagic deficits and altered function of c-Abl kinase, while other brain regions (hippocampus, amygdala and cortex) show normal c-Abl and autophagy. We hypothesize that these selective events in the VTA likely correlate with an impairment of Aβ clearance and with the observed neurodegeneration events. Moreover, it is also possible to hypothesize that the selectivity in VTA DAergic degeneration in our model likely arises from a dying-back mechanism starting from the axonal terminals in projection area, and that autophagic dysfunctions

that occur in DAergic VTA neurons contribute to making these cells more vulnerable. We now also include this idea in the Discussion (page 17).

Nobili, A., Latagliata, E.C., Viscomi, M.T., Cavallucci, V., Cutuli, D., Giacovazzo, G., Krashia, P., Rizzo, F.R., Marino, R., Federici, M., et al. (2017). Dopamine neuronal loss contributes to memory and reward dysfunction in a model of Alzheimer's disease. Nat. Commun. 8, 14727.

2. The extracellular accumulation of Aβ in Tg mouse models or direct injections of Aβ into the forebrain areas has been shown to reduce TH terminals that would be involved in retrograde VTA cell abnormalities and even cell death in the VTA. I think the authors should discuss these possibilities.

The Referee is correct. The hypothesis that the selectivity in VTA DAergic degeneration in our model likely arises from a dying-back mechanism starting from the axonal terminals in projection areas is now presented further in the Discussion (page 17).

3. The reduced activity of another prominent catecholaminergic structure in the early stages of AD is the locus coeruleus. It would be possible that this structure had cellular alterations similar to those of the VTA. Furthermore, damage or photoinhibition of LC has recently been shown to be related to DA hippocampal-dependent cognitive dysfunctions. It would be essential to see in the article a discussion about these possibilities.

We agree with the Reviewer concerning the LC-TH^+ projections to the hippocampus and had considered this possibility early on in our study. Yet, previously (Nobili et al., 2017) we demonstrated that in 6-month-old Tg2576 mice there is no change in the number of LC-TH^+ cells that could argue for degeneration in this area, nor any changes in the levels of noradrenaline in the hippocampus, while at the same age the VTA is well into advanced degeneration. Of course, changes in LC neurons might be functional, to reflect the drop of DA in the hippocampus and the DA-dependent memory problems. Therefore, since the majority of TH inputs to the hippocampus indeed come from the LC (Smith and Greene, 2012; Takeuchi et al., 2016) and older Tg2576 mice are reported to show deficits in the LC (Guérin et al., 2009), it is plausible that the DA hippocampal- dependent cognitive dysfunctions might be the combined result of VTA DA neuron degeneration and reduced axonal release from LC-TH^+ neurons (even though the cell bodies in the LC still appear intact). This is in line with the hypothesis of a dying-back mechanism of TH^+ terminals in AD, initiated in the projection areas affected by Aβ. We now discuss these ideas further in the Discussion (page 17).

Guérin, D., Sacquet, J., Mandairon, N., Jourdan, F., and Didier, A. (2009). Early locus coeruleus degeneration and olfactory dysfunctions in Tg2576 mice. Neurobiol. Aging 30, 272–283.

Nobili, A., Latagliata, E.C., Viscomi, M.T., Cavallucci, V., Cutuli, D., Giacovazzo, G., Krashia, P., Rizzo, F.R., Marino, R., Federici, M., et al. (2017). Dopamine neuronal loss contributes to memory and reward dysfunction in a model of Alzheimer's disease. Nat. Commun. 8, 14727.

Smith, C.C., and Greene, R.W. (2012). CNS dopamine transmission mediated by noradrenergic innervation. J. Neurosci. 32, 6072– 6080.

Takeuchi, T., Duszkiewicz, A.J., Sonneborn, A., Spooner, P.A., Yamasaki, M., Watanabe, M., Smith, C.C., Fernández, G., Deisseroth, K., Greene, R.W., et al. (2016). Locus coeruleus and dopaminergic consolidation of everyday memory. Nature 537, 357–362.

Reviewer #2:

This is a very interesting manuscript showing that Nilotinib, a c-Abl inhibitor, may protect VTA neurons in Tg2576 mouse model and attenuate cognitive deficit. The study is a reasonable extension of prior studies from this group that show loss early loss of VTA dopaminergic neurons contribute to deficits in Tg2576 model of amyloid pathology. In the current manuscript, significant aspect of the manuscript is focused on additional description of the dopaminergic deficits in the Tg2576 mice. While these are all very interesting data, it does not contribute sufficiently to understanding the role of c-Abl in neurodegeneration or AD pathogenesis. Overall, the authors need to provide additional experiments regarding the specificity and the mechanisms of c-Abl and nilotinib. Following specific issues, if addressed will improve the manuscript.

1. Figures 1, 2, and 3 are very nice description of the e-phys deficits of VTA DA neurons. However, the results are only peripherally relevant to the title of the manuscript. Thus, this data should be minimized and summerized into one figure

According to the Reviewer's suggestion, Figures 1, 2, and 3 are now summarized into two main figures. We rationalized Supplementary Figures 1 and 2 accordingly, reporting basic electrophysiological parameters in a Supplementary Table 1 and moving the analysis of dopaminergic currents at 1- and 3-months of age to Supplementary Figure 2. This way, Figure 2 is more straightforward, reporting only data regarding 6 months of age along with GABA recordings at 3 months of age. We believe that although these data are not directly relevant to the title, they give an idea, together with the morphological analysis, of how the dysfunction in c- Abl and autophagy is reflected into the function and morphology of the neuron.

2. Figure 4 is also an important data but it is more of an extension of prior study and not directly related to how c-Abl might be acting to cause loss of DA neurons.

We thank the Reviewer for this comment. We believe that functional and morphological analysis are of fundamental importance to disentangle the processes that occur during the disease progression. Thus, we measured soma perimeter and area of DAergic neurons at the beginning of neurodegeneration occurring in VTA. In 6-month-old Tg2576 a subpopulation of these cells (lateral VTA) showed an altered morphology that could correlate with the ongoing cell loss. We reevaluate these parameters upon Nilotinib treatment to investigate whether the improvement of cell viability was correlated with a recovery of cell morphology.
Interestingly, we observed that cell viability is accompanied by a morphological recovery and by an improvement in cell functions.

3. Authors need to provide summery of actual number and statistical parameters associated with data in Figure 5 (B, C, D).

We thank the Reviewer for his/her comment. However, the statistical parameters and the number of neurons analyzed were already present in the figure legend (now Figure 4).

4. I am not convinced that the effects of nilotinib is directly on the VTA neurons. First, authors need to show what is the status of c-Abl and autophagy in other brain regions, such as hippocampus, cortex, and amygdala. Further, authors need to determine whether nilotinib is impacting APP and Abeta levels in multiple brain regions. Without these data, there is no evidence that nilotinib is acting directly on dopaminergic neurons. Perhaps the authors could also provide immunolocalization of c-Abl activation in VTA neurons (e.g. Karim et al., Mol Neurodegen. 2020).

In response to the Reviewer's request and according to this suggestion, we analyzed in 3-month-old Tg2576 and age-matched control mice the c-Abl status in several brain regions, such as hippocampus, amygdala, and cortex. In all these areas we observed no differences in both basal and phosphorylated levels, indicating no alterations in its activity in these areas (new Supplementary Figure 3C-E). Thus, it appears that c-Abl activity is increased only in the midbrain region (Figure 4E). We also analyzed autophagy by measuring LC3 conversion (LC3I to LC3II) by immunoblot analysis in all the above-mentioned areas. As above, we observed an increase of LC3II/LC3I ratio only in the midbrain (new Figure 4E and Supplementary Figure 3C-E), indicating a selective accumulation of autophagosome vesicles in this area. This data is perfectly in line with TEM and confocal microscopy observations.
To determine whether Nilotinib reduces Aβ load, we quantified Aβ levels by immunofluorescence using the 6E10 antibody in different neuronal populations from several brain regions (VTA, hippocampus,

amygdala, cortex). Interestingly, we observed a significant reduction of this protein in all neurons from the examined areas (new Figure 5C and Supplementary Figure 4C-D), indicating a global effect of Nilotinib in Tg2576 mice. This of course indicates that Nilotinib does not have a selective action only in VTA neurons, in line with the fact that treatment was systemic.

We interpret our results to mean that, although neurons in other brain areas, at least at this stage, do not appear to suffer from the Aβ increase, VTA neurons start to degenerate (together with undergoing functional and morphological changes), likely due to the deficits in autophagy that would worsen the health of the neuron and/or accelerate the accumulation of Aβ. In this line of thinking, Nilotinib rescues autophagy in DA neurons and prevents degeneration. In the Discussion (page 17), we now hypothesize that the dopaminergic neurons in the VTA are more susceptible than others to cell death and the Aβ overexpression and autophagy dysfunction cumulatively contribute to their susceptibility. Moreover, we discuss that although the pharmacological treatment promotes Aβ clearance in several brain regions, it directly acts on dopaminergic neuron by preventing their dysfunction and rescuing both their functionality, morphology, and survival.

According to the reviewer's suggestion, we also tried to perform immunolocalization of phosphorylated form of c-Abl in VTA dopaminergic neurons. Unfortunately, despite repetitive attempts, the staining obtained is not very reliable as it seems not specific (we observe mainly a nuclear staining). We provide representative images at the bottom of this file, for the referee to judge as well.

Since we were unable to obtain reliable phospho-c-Abl staining, we performed immunohistochemistry and immunofluorescence of the total form of c-Abl, to understand if the modifications that we observed involve the dopaminergic neurons of the VTA. By immunohistochemistry we observed that the antibody against total c-Abl significantly marks neurons of the midbrain, particularly those of the VTA (Supplementary Figure 3A). We also confirmed that c-Abl is expressed in DA neurons by co-labelling c-Abl and TH^+ VTA (Supplementary Figure 3B). Thus, we are confident that the modification of the activated levels of c-Abl that we quantified by western blot from midbrain lysates is strictly related to modification in the phosphorylation status of c-Abl occurring in VTA DA neurons.

5. Authors should also provide evidence that nilotinib treatment results in inhibition of c-Abl and alleviated the autophagy deficits seen in Figure 5.

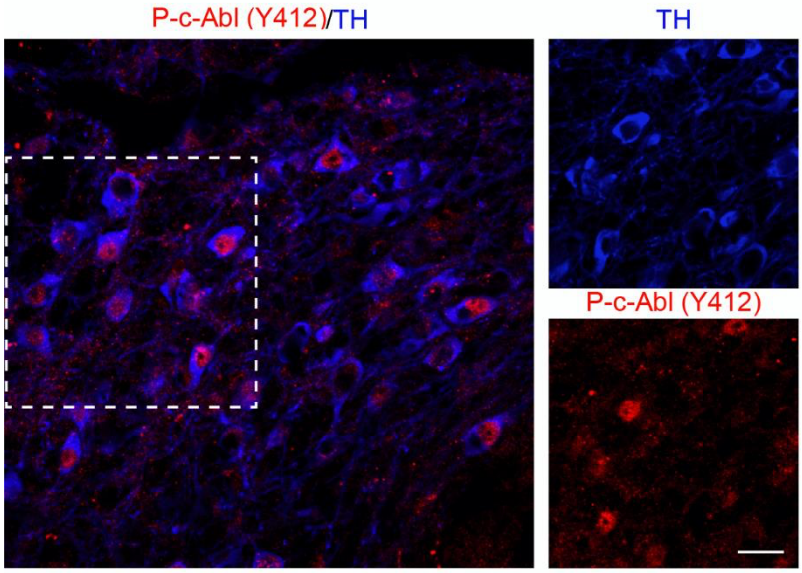
We thank the Reviewer for the recommendations as the new experiments greatly improve the quality of our work (please see also reply to comment 4). As requested, we analyzed c-Abl activity in the midbrain from 3- month-old Tg2576 mice treated with 1 mg/Kg of Nilotinib or saline. As expected, we observed a reduction of phosphorylated levels of c-Abl, indicating a reduction of its activity (Fig. 5A).

Furthermore, to investigate whether Nilotinib alleviates autophagy deficits, we analyzed LC3 conversion by immunoblot analysis. Nil-treated Tg mice showed a reduction of LC3-II/LC3-I ratio (Fig. 5A), indicating a reduction in autophagosome vesicles in midbrain neurons, and improvement in autophagy. Moreover, we quantified the number and the dynamics of autophagic vesicles in Tg2576 mice injected with the AAV2/9- mCherry-GFP-LC3 construct upon Nilotinib treatment. In the DAergic neurons we observed that Nilotinib induces a reduction in autophagosomes accumulation, improving autophagic flux (Fig. 5B).

Finally, we modified the Box and Whisker plot of Figure 4D (old Fig.5) because we realized that it contained wrong values. However, the interpretation of data remains invariant. We apologize for the mistake.

6. The text needs to be revised as many statements are too wordy and with non-standard use of terms.

We revisited the text as suggested and made changes at different points.



Representative confocal images showing immunofluorescent labelling for TH (blue) and P-c-Abl (Y412) (red) in VTA coronal sections from 3- month-old WT mice (scale: 20 µm).

2nd Decision Letter

Dear Prof. D'Amelio,

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.

To submit your revised manuscript go to https://www.editorialmanager.com/proneu/ and log in as an Author where you will see a menu item called 'Submission Needing Revision'.

Please resubmit your manuscript by Apr 11, 2021.

We look forward to receiving your revised manuscript.

Kind regards,

Jeannie Chin
Associate Editor
Progress in Neurobiology

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Comments from the Editors and Reviewers:

Reviewer #1: I believe that the authors have addressed all comments and the revised manuscript has been improved.

Reviewer #3: This is an interesting work that adds new information about the dopamine system alterations in AD pathogenesis. The work is well-performed, the experiments are well designed and the authors responded adequately to the reviewer's concerns, but some elements diminish my enthusiasm for its publication. I think that the novelty of the work is not so high, it is a rather correlational study and has some results coherence problems that have to be corrected or explained. However, I think that if the data consistence problems were amended or correctly explained, the paper could be suitable for publication.

Respect the novelty; the paper evaluates two main issues that previously have been explored. The first issue studied is the alterations of dopaminergic neurons at the ventral tegmental area (VTA) of AD models. It is relevant because alterations in the dopaminergic system have been linked to memory deficits in AD patients. Thus, this is the continuation of previous group work in which the authors described age-dependent DAergic neurons loss at VTA of Tg2576 AD mouse model. DAergic neurons loss would results in lower DA outflow in the hippocampus contributing to CA1 synaptic plasticity and memory impairments in AD. Here the authors characterized in more detail the changes in VTA DAergic neurons of Tg2576 mice. The new information added here is that the DAergic neurons show hiperexcitability and morphological changes in lateral VTA area. Then, although this data is new its novelty is not so high.

The second issue is the role of c-Abl activation and dysfunctional autophagy in AD pathology. Here is showed that these changes would be happening earlier in the VTA DAergic neurons respect other brain areas. Thus in agreement with previous results in PD and AD models, they also show that chronic treatment of Tg2576 mice with Nilotinib reduces c-Abl phosphorylation and improves autophagy flux. This correlated with a reduction of Aβ levels in lateral VTA, a reduction in functional and morphological alterations of lateral VTA DAergic neurons and, cognitive deficits decrease. The c-Abl activation and dysfunctional autophagy in VTA have not been described previously in the early stages of AD, but the effects of Nilotinib have been previously evaluated in AD pathology.

Overall it is a well-done study but mainly correlational without a clear mechanism to explain the observed changes. For example the changes in DAergic neurons excitability correlated with morphological alterations and a decrease in TH+ neurons number, but it is not clear which is the cause of this increased excitability. It is a consequence of DA neurons damage or loss? or the exacerbated excitability promotes the loss of cells?. The authors showed autophagy dysfunction and c-Abl activation but the mechanisms involved are discussed but not studied. Indeed Nilotinib promotes autophagy and reduced the Ab accumulation.

The paper is well written, the methodology, results, and statistical analysis are clearly described, and figures and graphs are well performed. In general, the results are good and clear but present a few consistency problems that have to be improved.

In the following section, the results will be analyzed in more detail and discuss if they support the conclusions/statements according the highlights or subtitles.
Highlights.
1.- Tg2576 DA neurons show increased excitability and changes in neuronal conductances.
Yes, the data presented here support that the DAergic neurons in VTA of Tg2576 AD mice have increased excitability. The electrophysiological data is nice and clearly shows that DAergic neurons of 6-month Tg2576 mice have increased frequency of spontaneous firing, increased excitability with decreases in the threshold for AP, and increases of the number of AP at different depolarizing currents (Figure 1).
The authors evaluated if changes in SK, Ih, Im currents or decreases in inhibitory GABAergic activity could explain the increased DAergic neurons excitability. Nonetheless, the mechanisms linking the hyperexicitablity with DAergic neurons degeneration in Tg2576 remain to be defined. The authors discuss some plausible mechanisms but not were demonstrated.

Although the data of 6-month Tg2576 mice clearly show increased excitability of VTA DAergic neurons, the data for 3-months AD mice are less clear. At 3 months of age, the Tg2576 showed only a slight increase of VTA DAergic neurons spontaneous firing frequency (Figure 1a). The spontaneous firing frequency shows high variability and the rise, from ~3.0 to ~3.2, is very small. The difference seems to be statically significant but is not clear if in the statistical analysis the n used is the neurons number or the mice number.
With this data, the authors sustain that the change in DAergic excitability is as early as 3 months and use the same age to study the effects of Nilotinib in spontaneous firing frequency (Figure 6). Here the Tg2576 mice spontaneous firing frequency was ~4.1 dropping to ~3.0 in Tg2576 treated with Nilotinib. Please explain why the spontaneous firing frequency of Tg2576 DA neurons changed?. Does this affect the significance of the previous results (Figure 1) and the Nilotinib effects?

2 • Morphology is altered in VTA DA neurons of Tg2576 mice.
This statement is only partially sustained by the data. Again the problem here is related to the data consistency. The soma area and soma perimeter values for DAergic neurons of Tg2576 and WT mice in Figure 3 are not similar to the observed in Figure 6C.

First I want to indicate that Figure 3 seems to have a mistake. The same TH+ neuron photo is displayed twice in different conditions. The TH+ cell showed for intermediate VTA of Tg2576 mice (Figure 3B) is the same neuron that is showed as TH+ cell for intermediate VTA of WT mice (Figure 3C).

Concerning to values inconsistencies in Figure 3C, the soma area of the TH+ cells at lateral VTA of 6 months old WT mice was ~ 125 um2 and decreased to ~ 98 um2 in Tg2576 mice (this decrease was significant). In Figure 6 the same evaluation, the soma area of TH+ cells in lateral VTA, was performed in the same Tg2576 mice at the same age. However here the soma area of TH+ cells of WT mice was ~ 200 um2 and decreased to ~ 125 um2. This last value is the same that the soma area for WT mice in Figure 3C?. Then which is the real soma area of TH+ cells in 6 months old Tg2576 and WT mice?. Which is the real soma area decrease, 25 um2 (Figure 3C) or 75 um2 (Figure 6c)?.

The same problem happens when we compared the data for the Soma perimeter of TH+ cells in Figure 3C and Figure 6C. The soma perimeter of TH+ positive cells in lateral VTA of 6 months old WT mice was 44 um and decreased to 37 um in Tg2576 mice. In Figure 6C the soma perimeter TH+ cells in the lateral VTA of WT mice was 57 and diminished to 45 um in Tg2576 mice, a value very similar to WT in Figure 3C?. Then which is the real soma perimeter of DAergic cells in 6 months old Tg2576 and WT mice?.

This variability on the morphological parameters has to be explained, and although seem not affects the conclusion that DAergic present a reduction in Tg2576 weakens its and the significance of the Nilotinib effects.

A similar problem is observed in the TH+ cells total number of VTA both for WT and Tg2576 mice. Here the numbers are lower than the numbers that the authors showed in previous work (Nobili et al., 2017). It is necessary to discuss these differences. In this line, around 4.000-5.000 TH+ cells are lost in total VTA of Tg2576, TH+ cell numbers of intermediate VTA not suffer significant changes, but the reduction of TH+ cells in lateral VTA is only around 500-600?. How explain this discrepancy? I understand that the numbers could vary in a certain range but the differences do not match.

3. Autophagy alterations in DA neurons in the VTA of Tg2576 mice

Yes, the data sustain that DAergic cells in VTA of the 3-month old Tg2576 mice show autophagy impairments. The increases of yellow dots and the LC3 II levels are consistent with an autophagy flux

reduction. Also interestingly was observed the selective VTA activation of the c-Abl kinase at this age. However, the graph for autophagosome density quantification by TEM has to be improved because is not clear the difference in the media. The statistical analysis has to be revised and indicates if the n that was used is the number of neurons or mice?. The statistical power increased with high number de cells but each neuron is not a different experiment.

4. The c-Abl inhibitor Nilotinib prevents autophagy deficits and reduces Aβ load

Yes, the data presented supports these statements. 6-months Tg2576 mice treated with Nilotinib showed a reduction of c-Abl activation and the LC3 II in the VTA compared with the Tg2576 no treated. Also, Tg2576 mice treated with Nilotinib showed a lower number of yellow dots in TH+ cells (corresponding to the autophagosomes) than the Tg2576 without treatment. Unfortunately, the autophagy followed by IF (yellow dots) and by LC3 II levels in Tg2576 and Tg2576 + Nilotinib mice (Figure 5) and the WT and Tg2576 mice (Figure 4) were analyzed independently and not compared between them. Because of that, it is not possible to confirm that the autophagy alterations are prevented, e.i. in Nilotinib treated Tg2576 mice the values are not significantly different from those of WT mice, and thus say that the autophagy deficit was prevented.
On other hand, the reduction of intracellular Ab signal in the VTA cells of Tg2576 mice when treated with Nilotinib followed by IF, supports the Ab load reduction.

5. Nilotinib rescues DA neuron degeneration, hippocampal DA levels, and memory function

Yes, the data support this conclusion, the authors showed that Nilotinib reduces the frequency of spontaneous firing, indicating a reduction of the excitability of the DAergic neurons observed in the 3-month old Tg2576 mice and Nilotinib increased the area and perimeter of the DAergic cells soma of 6-months old Tg2576 mice, although morphology was not totally recovered.
Although how mentioned above, these results have to be analyzed in the context of the Figures 1 data for results coherence, these results themselves are consistent and clear. The appropriate control groups are incorporated and the observed prevention of VTA DAergic neurons degeneration is consistent with the increased levels of DA in the hippocampus. In agreement with the previous results, the increase in the hippocampus DA levels in the AD mice treated with Nilotinib was associated with better performance on the NOR test. Although Nilotinib effects on memory are probably associated with VTA degeneration reduction also effects in other brain areas could contribute, the authors discuss it.

Although the study is mainly correlational the potential mechanisms are well discussed including previous works and literature, this made this work interesting and appealing.

Minor points,

Some English mistakes in spelling, words use and, grammar. For example:

page 3 Introduction: line 7 The amyloid hypothesis -that individuates the accumulation and deposition of oligomeric or fibrillar Aβ .... the sentence is not clear

page 3 Introduction: line 20 neurotransmission or pharmacological manipulations that increase the DA drive have been shown to improve.... the sentence is not clear

2nd Author Response Letter

We would like to thank the Reviewers for all their comments during the entire reviewing process. Here is a reply to the last points:

Reviewer #1: I believe that the authors have addressed all comments and the revised manuscript has been improved.

We thank the Reviewer for the help and suggestions during the revision process.

Below, we provide a point-by-point reply to all the comments raised by **Reviewer #3**; in the main text all the relevant changes are now highlighted for easiness of reading.

This is an interesting work that adds new information about the dopamine system alterations in AD pathogenesis. The work is well-performed, the experiments are well designed and the authors responded adequately to the reviewer's concerns, but some elements diminish my enthusiasm for its publication. I think that the novelty of the work is not so high, it is a rather correlational study and has some results coherence problems that have to be corrected or explained. However, I think that if the data consistence problems were amended or correctly explained, the paper could be suitable for publication.

Respect the novelty; the paper evaluates two main issues that previously have been explored. The first issue studied is the alterations of dopaminergic neurons at the ventral tegmental area (VTA) of AD models. It is relevant because alterations in the dopaminergic system have been linked to memory deficits in AD patients. Thus, this is the continuation of previous group work in which the authors described age-dependent DAergic neurons loss at VTA of Tg2576 AD mouse model. DAergic neurons loss would results in lower DA outflow in the hippocampus contributing to CA1 synaptic plasticity and memory impairments in AD. Here the authors characterized in more detail the changes in VTA DAergic neurons of Tg2576 mice. The new information added here is that the DAergic neurons show hiperexcitability and morphological changes in lateral VTA area. Then, although this data is new its novelty is not so high.

These are considerations that we ourselves made when submitting this work. Yet, we beg to disagree with the Reviewer concerning novelty: here, we do not simply confirm our previous results concerning the degeneration of DA neurons in AD but prove a series of important alterations in these neurons, at different stages of the disease, that can correlate with degeneration. Our aim was (and is) to understand as much as possible the basis of this degeneration, similarly to what has been done for many years with Parkinson's studies: researchers know that DA neurons degenerate in the substantia nigra (SNpc) but they keep on studying them from different points of view in order to understand (and therefore hopefully combat) the degeneration.
We also reiterate the novelty of the current work as it identifies very early pathological changes in dopamine neurons from the VTA. This VTA "changes" should be considered as prodromal markers to be validated in the human disease. Thus, our novelty lies in the fact that ours is, to our knowledge, the

first study to analyze the morphological/autophagy/functional changes in DA neurons in a model of early AD.

The second issue is the role of c-Abl activation and dysfunctional autophagy in AD pathology. Here is showed that these changes would be happening earlier in the VTA DAergic neurons respect other brain areas. Thus in agreement with previous results in PD and AD models, they also show that chronic treatment of Tg2576 mice with Nilotinib reduces c-Abl phosphorylation and improves autophagy flux. This correlated with a reduction of Aβ levels in lateral VTA, a reduction in functional and morphological alterations of lateral VTA DAergic neurons and, cognitive deficits decrease. The c-Abl activation and dysfunctional autophagy in VTA have not been described previously in the early stages of AD, but the effects of Nilotinib have been previously evaluated in AD pathology.

We confirm that the effects of Nilotinib have been previously evaluated in AD pathology but the evidence (from our work) that Nilotinib might be effective in very early stages make tyrosine kinase inhibitors good candidates to be clinically explored in prodromal phase. This point is very relevant because the general consensus about the failure of experimental therapies in AD is that the drug treatment is delivered when the disease is already in an advanced stage.

Overall it is a well-done study but mainly correlational without a clear mechanism to explain the observed changes. For example the changes in DAergic neurons excitability correlated with morphological alterations and a decrease in TH+ neurons number, but it is not clear which is the cause of this increased excitability. It is a consequence of DA neurons damage or loss? or the exacerbated excitability promotes the loss of cells?. The authors showed autophagy dysfunction and c-Abl activation but the mechanisms involved are discussed but not studied. Indeed Nilotinib promotes autophagy and reduced the Ab accumulation.

With regards to the increased excitability of the VTA DA neurons in the Tg2576 mouse, unfortunately we could not provide a clear answer as to why these neurons are hyperexcitable, or what the basis of this alteration is. This is despite the fact that we investigated the most frequently studied (and most important) conductances related to these neurons, as well as their inhibitory input. Unfortunately, there are limits to the information that a single technique such as electrophysiology can provide without interfering with the nature of the neuron, and other techniques that might provide essential information on the status of these neurons (such as 2-photon analysis of dendritic calcium dynamics) go beyond our technical expertise. Nonetheless, we thoroughly discuss in the Discussion section (pag. 14-15) some

factors that could contribute to the increased excitability, including changes in K+ or Na+ conductances, increased Aβ or altered autophagy that might affect the functioning/expression of voltage-gated channels. Of note, similar problems in defining the reasons underlying functional changes arise when one studies the electrophysiology of DA neurons in models of Parkinson's disease: many different deficiencies are described, yet it is still not clear if these are the result or the culprit of the ongoing degeneration process. Additionally, the variability that highly characterizes these cells in the midbrain[1-3] makes the situation even more complicated to disentangle because subtle changes in electrophysiological properties can be 'lost' when cells are pooled together, and this is typically one of the reasons why these neurons are particularly difficult to study. Nonetheless, the theory we discuss in the Discussion (the 'stressful pacemaker theory'[4,5]), used as a probable explanation for the degeneration of SNpc DA neurons in Parkinson's, is a very tempting theory to also explain the degeneration of VTA neurons in AD.

1. Lammel, S., Hetzel, A., Häckel, O., Jones, I., Liss, B., Roeper, J., 2008. Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. Neuron 57, 760–773. https://doi.org/10.1016/j.neuron.2008.01.022
2. Roeper, J., 2013. Dissecting the diversity of midbrain dopamine neurons. Trends Neurosci. 36, 336–342. https://doi.org/10.1016/j.tins.2013.03.003
3. Ungless, M. and Grace, A., 2012. Are you or aren't you? Challenges associated with physiologically identifying dopamine neurons. Trends Neurosci. 35, 422–430. https://doi.org/10.1016/j.tins.2012.02.003
4. Chan, C.S., Gertler, T.S., Surmeier, D.J., 2009. Calcium homeostasis, selective vulnerability and Parkinson's disease. Trends Neurosci. 32, 249–256. https://doi.org/10.1016/j.tins.2009.01.006
5. Sulzer, D., 2007. Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. Trends Neurosci. 30, 244–250. https://doi.org/10.1016/j.tins.2007.03.009

The paper is well written, the methodology, results, and statistical analysis are clearly described, and figures and graphs are well performed. In general, the results are good and clear but present a few consistency problems that have to be improved.

We thank the Reviewer for these comments on the quality of our work.

In the following section, the results will be analyzed in more detail and discuss if they support the conclusions/statements according the highlights or subtitles.

Highlights.

1- Tg2576 DA neurons show increased excitability and changes in neuronal conductances.

Yes, the data presented here support that the DAergic neurons in VTA of Tg2576 AD mice have increased excitability. The electrophysiological data is nice and clearly shows that DAergic neurons of 6-month Tg2576 mice have increased frequency of spontaneous firing, increased excitability with decreases in the threshold for AP, and increases of the number of AP at different depolarizing currents (Figure 1).

The authors evaluated if changes in SK, Ih, Im currents or decreases in inhibitory GABAergic activity could explain the increased DAergic neurons excitability. Nonetheless, the mechanisms linking the hyperexcitability with DAergic neurons degeneration in Tg2576 remain to be defined. The authors discuss some plausible mechanisms but not were demonstrated.

We confirm that the increased excitability is a consequence of an early DA neuron damage as demonstrated by the evidence that no alterations are detectable in younger animals, at 1 month of age. However, we cannot exclude that the increased excitability, as early pathological response, might contribute to the final death of dopamine neurons.

As we also discuss above, we investigated four different conductances (SK, Ih, Im, GABA currents) that are known to alter the excitability of DA neurons, yet even at 6 months of age we did not identify any important differences between WT and Tg neurons (we actually observed a reduction in the absolute amplitude of both Ih and SK current in Tg mice, which is lost when normalizing onto cell surface). Of note, the neurons we are able to study with electrophysiology in Tg mice are probably those that are more resistant to the degeneration process (roughly 60% of neurons at this age), since already dead or dying neurons would be technically impossible to patch. This, together with the high variability that characterizes these neurons in the VTA, make it more difficult to pinpoint specific alterations happening in Tg mice. Additionally, to identify the precise mechanism by which hyperexcitability is linked to degeneration is not an obvious thing to do (see also for example the numerous studies using Parkinson's models), since many different pathways can be involved concurrently. For example, is the Ca^{2+} homeostasis of these neurons altered due to the increased firing? We considered this possibility but the fact that the SK current density appears to be unchanged in Tg neurons points against this hypothesis since SK channels are the 'natural Ca^{2+} sensors' of dopamine neurons. Additionally, VTA DA neurons do not seem to have an activated Ca^{2+} conductance during pacemaking, unlike neurons in the SN[6].

We are more tempted to believe that the increased excitability of these neurons is more likely due to the autophagy changes occurring in these cells, which are expected to alter the functioning, recycling and/or distribution of channels on the membrane surface, thus altering cell firing. Changes in the

morphology of the neuron would also be expected to alter the distribution of channels in the soma and dendrites, with very plausible effects on the excitability of the cell. Additionally, hyperexcitability might be a result of Aβ interference with Na-channels as in the case of pyramidal neurons[7], as we discuss in the Discussion. Of course, all these considerations merit further investigations that however go beyond the purpose of our study and could be the object of many different manuscripts in the future. We would also like to point out that most of these experiments are more easily performed in neurons in culture and not in brain slices, which *per se* complicates matters even further.

6. Khaliq, Z.M., Bean, B.P., 2010. Pacemaking in dopaminergic ventral tegmental area neurons: depolarizing drive from background and voltage-dependent sodium conductances. J Neurosci. 30, 7401-7413. https://doi.org/10.1523/JNEUROSCI.0143-10.2010
7. Ciccone, R., Franco, C., Piccialli, I., Boscia, F., Casamassa, A., de Rosa, V., Cepparulo, P., Cataldi, M., Annunziato, L., Pannaccione, A., 2019. Amyloid β-Induced Upregulation of Na v 1.6 Underlies Neuronal Hyperactivity in Tg2576 Alzheimer's Disease Mouse Model. Sci. Rep. 9, 13592. https://doi.org/10.1038/s41598-019-50018-1

Although the data of 6-month Tg2576 mice clearly show increased excitability of VTA DAergic neurons, the data for 3-months AD mice are less clear. At 3 months of age, the Tg2576 showed only a slight increase of VTA DAergic neurons spontaneous firing frequency (Figure 1a). The spontaneous firing frequency shows high variability and the rise, from ~3.0 to ~3.2, is very small. The difference seems to be statically significant but is not clear if in the statistical analysis the n used is the neurons number or the mice number.
With this data, the authors sustain that the change in DAergic excitability is as early as 3 months and use the same age to study the effects of Nilotinib in spontaneous firing frequency (Figure 6). Here the Tg2576 mice spontaneous firing frequency was ~4.1 dropping to ~3.0 in Tg2576 treated with Nilotinib. Please explain why the spontaneous firing frequency of Tg2576 DA neurons changed? Does this affect the significance of the previous results (Figure 1) and the Nilotinib effects?

Indeed, the high variability in the spontaneous firing (Figure 1a) is a direct indication of the heterogeneity in this population of neurons, which, as mentioned above, contributes to 'missing' subtle changes in neuronal properties. In fact, the changes in the neuronal excitability are much more subtle at 3 months of age, compared to older mice (6 months), in line with the fact that at this time point the neurons are only just starting to degenerate. It is also in line with the fact that younger Tg neurons (1 month of age) show a firing very similar to age-matched WT mice, despite the large number of neurons tested.

For the statistical analysis in all the figures, the n used is the number of data points shown in the figure, thus, for Fig.1a the n is the number of neurons, as it is standard practice for electrophysiology to consider each neuron as an independent subject, irrespective of the animal of origin. This takes into account the consideration that if neurons are pooled together based on gender, genetic background and age, there shouldn't be differences in their functioning.

The reviewer is correct to say that the spontaneous firing frequency of Tg neurons treated with saline (Tg sal, ~4.1 Hz) is slightly higher compared to that of neurons from naïve Tg animals (~3.0 Hz). Yet, we do not think it is appropriate to directly compared treated vs non treated mice. The frequent manipulation of the mice and the treatment regime we used (i.p injections every other day for a 1.5 month) can be stressful for the animal, with indirect consequences on neuronal circuits and neuronal function that can be seen as changes in the basal firing activity of neurons. Additionally, our Tg sal animals received saline containing 5% DMSO, to match the solvent content of nilotinib-treated mice, and many reports indicate that DMSO, even as low as 5%, can have various effects on neuronal excitability by acting on glutamatergic or GABAergic channels[8,9]. However, to avoid possible misunderstanding, we replace "saline" with "vehicle" in the text, figures, and legends.

As to why the spontaneous firing is reduced in mice treated with nilotinib, we hypothesize that this is due to the ability of the drug to improve the autophagic process and cell morphology. As mentioned above, it is logical to hypothesize that alterations in the autophagic process and morphology of the neuron would be reflected in alterations in the functioning, recycling and/or distribution of channels on the cell membrane, and thus to changes in neuronal firing in Tg neurons. Our data showing improvement of firing with nilotinib are in line with our hypothesis that the stressful condition in which a neuron is found when autophagy is deficient can be reflected as an increase in neuronal excitability.

8. Lu, C., Mattson, M.P., 2001. Dimethyl sulfoxide suppresses NMDA- and AMPA-induced ion currents and calcium influx and protects against excitotoxic death in hippocampal neurons. Exp Neurol. 170, 180-185. https://doi.org/10.1006/exnr.2001.7686
9. Nakahiro, M., Arakawa, O., Narahashi, T., Ukai, S., Kato, Y., Nishinuma, K., Nishimura, T. 1992. Dimethyl sulfoxide (DMSO) blocks GABA-induced current in rat dorsal root ganglion neurons. Neurosci Lett. 138, 5- 8. https://doi.org/10.1016/0304-3940(92)90459-K

2- Morphology is altered in VTA DA neurons of Tg2576 mice.

This statement is only partially sustained by the data. Again the problem here is related to the data consistency. The soma area and soma perimeter values for DAergic neurons of Tg2576 and WT mice in Figure 3 are not similar to the observed in Figure 6C.

First I want to indicate that Figure 3 seems to have a mistake. The same TH+ neuron photo is displayed twice in different conditions. The TH+ cell showed for intermediate VTA of Tg2576 mice (Figure 3B) is the same neuron that is showed as TH+ cell for intermediate VTA of WT mice (Figure 3C).

Concerning to values inconsistencies in Figure 3C, the soma area of the TH+ cells at lateral VTA of 6 months old WT mice was ~ 125 um2 and decreased to ~ 98 um2 in Tg2576 mice (this decrease was significant). In Figure 6 the same evaluation, the soma area of TH+ cells in lateral VTA, was performed in the same Tg2576 mice at the same age. However here the soma area of TH+ cells of WT mice was ~ 200 um2 and decreased to ~ 125 um2. This last value is the same that the soma area for WT mice in Figure 3C? Then which is the real soma area of TH+ cells in 6 months old Tg2576 and WT mice? Which is the real soma area decrease, 25 um2 (Figure 3C) or 75 um2 (Figure 6c)?

The same problem happens when we compared the data for the Soma perimeter of TH+ cells in Figure 3C and Figure 6C. The soma perimeter of TH+ positive cells in lateral VTA of 6 months old WT mice was 44 um and decreased to 37 um in Tg2576 mice. In Figure 6C the soma perimeter TH+ cells in the lateral VTA of WT mice was 57 and diminished to 45 um in Tg2576 mice, a value very similar to WT in Figure 3C? Then which is the real soma perimeter of DAergic cells in 6 months old Tg2576 and WT mice?

This variability on the morphological parameters has to be explained, and although seem not affects the conclusion that DAergic present a reduction in Tg2576 weakens its and the significance of the Nilotinib effects.

We thank the reviewer for the comments and apologize for the mistake in the Figure. **Figure 3B** is now corrected.

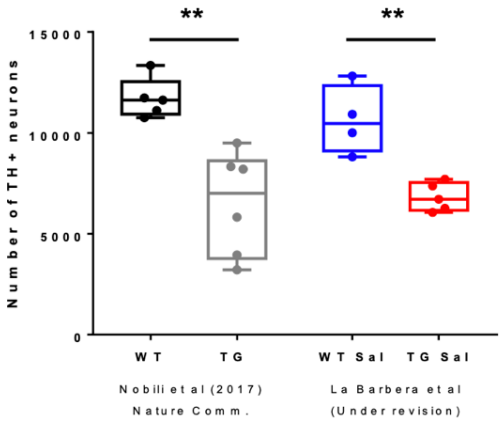
Concerning the discrepancy in morphology (soma area and perimeter), as argued above, we do not think it is appropriate to directly compared treated vs non treated mice. These treated mice have been repeatedly manipulated and injected for 4.5 months; the manner by which animals are handled by researchers has been shown to affect also the brain of the animal, including neuronal function and behavior[10,11]. We assume that changes in morphology can be a result of this manipulation. Unfortunately, such effects of animal handling cannot be avoided, and this is why internal controls (in our case saline-treated WT and Tg mice) are needed for every experiment in which the animal has been treated differently to naïves.

10. Sorge, R.E., et al. 2014. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods. 11, 629-632. http://dx.doi.org/10.1038/nmeth.2935
11. https://www.nature.com/news/male-researchers-stress-out-rodents-1.15106

A similar problem is observed in the TH+ cells total number of VTA both for WT and Tg2576 mice. Here the numbers are lower than the numbers that the authors showed in previous work (Nobili et al., 2017). It is necessary to discuss these differences. In this line, around 4.000-5.000 TH+ cells are lost in total VTA of Tg2576, TH+ cell numbers of intermediate VTA not suffer significant changes, but the reduction of TH+ cells in lateral VTA is only around 500-600? How explains this discrepancy? I understand that the numbers could vary in a certain range but the differences do not match.

We thank the Referee for these comments.

In our previous paper (Nobili et al. 2017), the numbers shown in the bar graph refer to total number of cells in the VTA, containing both TH+ and TH- neurons. In our current manuscript (**Figure 6B**) we considered only TH+ cells. To make things clearer, here we provide an attached graph where we plotted the TH+ cells counted in Nobili et al. 2017, with the TH+ cells counted in this work, and we confirm the number of neurons is the same between WT naïve and WT Sal, and Tg naïve and Tg sal. Of note, these experiments in the two papers have been performed by different researchers, in order to confirm the original data and exclude any researcher bias.



Regarding the TH+ neuron numbers in the lateral VTA (Fig. 3D), the analysis was performed only in one hemisphere (now it is specified in the Material and Methods), so if we consider both hemispheres, the numbers would double. Still, it is true that the reduction of neurons in the lateral VTA is too small compared to the total VTA loss; yet, the lateral portion we analyzed is relatively small and the cells are much more interspersed in respect to the entire VTA, and this could explain the small reduction observed. Indeed, in the Discussion we argue that the overall ~40% reduction of DA neurons in 6-month-old Tg mice is likely the result of diffuse degeneration in more than one single subregion.

3- Autophagy alterations in DA neurons in the VTA of Tg2576 mice

Yes, the data sustain that DAergic cells in VTA of the 3-month old Tg2576 mice show autophagy impairments. The increases of yellow dots and the LC3 II levels are consistent with an autophagy flux reduction. Also interestingly was observed the selective VTA activation of the c-Abl kinase at this age. However, the graph for autophagosome density quantification by TEM has to be improved because is not clear the difference in the media. The statistical analysis has to be revised and indicates if the n that was used is the number of neurons or mice? The statistical power increased with high number of cells but each neuron is not a different experiment.

We thank the Reviewer for his/her comment. The statistical analysis was performed on density of autophagosomes (number/um^2). We have improved the autophagosome quantification by calculating the density of autophagosomes for each TH positive neuron. We report new statistical results (the density of autophagosomes, as requested). Accordingly, the chart of **Figure 4C** has been replaced by the new version where on Y axis the "density of autophagosomes (number/μm^2 per neuron)" is reported. The results are similar to before (Mann-Whitney test **p=0.0013).

4- The c-Abl inhibitor Nilotinib prevents autophagy deficits and reduces Aβ load

Yes, the data presented supports these statements. 6-months Tg2576 mice treated with Nilotinib showed a reduction of c-Abl activation and the LC3 II in the VTA compared with the Tg2576 no treated. Also, Tg2576 mice treated with Nilotinib showed a lower number of yellow dots in TH+ cells (corresponding to the autophagosomes) than the Tg2576 without treatment. Unfortunately, the autophagy followed by IF (yellow dots) and by LC3 II levels in Tg2576 and Tg2576 + Nilotinib mice (Figure 5) and the WT and Tg2576 mice (Figure 4) were analyzed independently and not compared between them. Because of that, it is not possible to confirm that the autophagy alterations are prevented, e.i. in Nilotinib treated Tg2576 mice the values are not significantly different from those of WT mice, and thus say that the autophagy deficit was prevented.
On other hand, the reduction of intracellular Ab signal in the VTA cells of Tg2576 mice when treated with Nilotinib followed by IF, supports the Ab load reduction.

We thank the Reviewer for this comment. However, as previously explained, we do not think it is appropriate to compare treated vs non treated mice. Yet, we agree that, given that both western blots and autophagosomes dots from naïve and treated mice were analyzed independently, it is not possible to confirm that the autophagy alterations are fully prevented by the Nilotinib treatment. In line with this, we decided to change the phrase " Nilotinib prevents..." with "Nilotinib ameliorates..." in the Highlights and Figure 5 Legend.

5- Nilotinib rescues DA neuron degeneration, hippocampal DA levels, and memory function

Yes, the data support this conclusion, the authors showed that Nilotinib reduces the frequency of spontaneous firing, indicating a reduction of the excitability of the DAergic neurons observed in the 3- month old Tg2576 mice and Nilotinib increased the area and perimeter of the DAergic cells soma of 6- months old Tg2576 mice, although morphology was not totally recovered.

Although how mentioned above, these results have to be analyzed in the context of the Figures 1 data for results coherence, these results themselves are consistent and clear. The appropriate control groups are incorporated and the observed prevention of VTA DAergic neurons degeneration is consistent with the increased levels of DA in the hippocampus. In agreement with the previous results, the increase in the hippocampus DA levels in the AD mice treated with Nilotinib was associated with better performance on the NOR test. Although Nilotinib effects on memory are probably associated with VTA degeneration reduction also effects in other brain areas could contribute, the authors discuss it.

Although the study is mainly correlational the potential mechanisms are well discussed including previous works and literature, this made this work interesting and appealing.

We thank the Reviewer for these comments.

Minor points

Some English mistakes in spelling, words use and, grammar. For example:

-page 3 Introduction: line 7 The amyloid hypothesis -that individuates the accumulation and deposition of oligomeric or fibrillar Aβ .... the sentence is not clear

-page 3 Introduction: line 20 neurotransmission or pharmacological manipulations that increase the DA drive have been shown to improve.... the sentence is not clear

We revisited the text as suggested and made changes at different points.