EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Ipsilateral corticotectal projections from the primary, premotor and supplementary motor cortical areas in adult macaque monkeys: a quantitative anterograde tracing study

Michela Fregosi & Eric M. Rouiller

Review timeline:

Submission date:	01 June 2017
Editorial Decision:	28 June 2017
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Editorial Decision:	21 August 2017
Revision received:	25 August 2017
Accepted:	06 September 2017

Editor: Helen Barbas

1st Editorial Decision 28 June 2017

Dear Prof. Rouiller,

Your manuscript was reviewed by three external reviewers as well as by the Section Editor, Dr. Helen Barbas, and ourselves.

The reviews collectively indicate that your experiments generated new and important information. However, there are several substantial issues that need to be clarified/resolved before we can consider your manuscript further for publication in EJN.

As you can see, the Reviewers appreciated your findings and their significance. They also provided a list of points to address in order to help focus and provide a functional context of your findings for a broad audience. One of the issues arising pertains to the normalization of the data, which is not adequately explained or justified and is subject to pitfalls. A brief significance statement in the abstract is needed. Discussion of your findings in the context of both the origin of the pathways in the cortex and their termination within different layers of the superior colliculus and their functional specialization will help point to the overall significance of the findings. In this context, old physiological studies (Abrahams and Rose, 1975) on converging responses from muscles that move the eyes and the neck in the superior colliculus of cats may be relevant. Please be consistent in the naming of your cases to allow cross referencing in your studies. It is necessary to break up long sentences for clarity and go over the manuscript carefully to correct grammatical and orthographic errors. Finally, please ensure that the reference list and in-text citations adhere to EJN guidelines.

If you are able to respond fully to the points raised, we would be pleased to receive a revision of your paper within 12 weeks.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 1 (Miguel Angel García-Cabezas, Boston University, USA)

Comments to the Author
The paper “Ipsilateral corticotectal projections from the primary, premotor and supplementary motor cortical

areas in adult macaque monkeys: a quantitative anterograde tracing study" traces coticotectal projections injecting the anterograde tracer BDA in the cortex of 9 monkeys. Recently, the authors have described corticobulbar projections using 7 of those monkeys.

The authors find a density gradient of corticotectal projections across motor areas. Corticotectal projections originated in premotor (PM) and supplementary motor (SMA) areas are denser than corticotectal projections originated in the primary motor area (M1).

The experiments are well designed and the findings are of interest for a general audience of neuroscientists. Here are some suggestions to improve the manuscript.

Major comments:
1.- The authors do not use unbiased stereological methods for the analysis of bouton density in corticotectal projections. In the methods they state that:

"…it was possible to visualize and chart every individual axonal bouton, en passant or terminal. In such a case, stereological techniques do not apply (e.g. Lavenex at al., 2000; Geuna 2000; Benes and Lange 2001)."

The fact that the entire population of boutons can be visualized does not exclude the use of unbiased stereological methods. Actually, if some of the labeled boutons could not be visualized then sterology could not be applied because not every bouton would have the same chances to be counted and the estimation would be biased.

The strength of stereology is that the population of particles (boutons) in the region of interest (superior colliculus) could be estimated with a known error. Even more, the number of sections analyzed and the interval between them would be taken into account to calculate the error and no further correction would be needed. The authors' choice for exhaustive plotting instead of stereology is legitimate and enough to show trends in corticotectal projections across motor areas, but stereological methods are the gold standard and they are not excluded because of the visualization of boutons.

2.- The authors normalize the number of BDA labelled axonal boutons by the corresponding number of BDA labelled corticospinal axons in the same animal at the level of the pyramid, multiplied by 1000. This part of the methods, which is key for the interpretation of the data could be better explained. Why multiply by 1000? In how many sections did the authors counted BDA labeled axons?

3.- The authors analyzed ipsilateral corticotectal projections but in the discussion they mention that few if any axonal terminal boutons were seen in the contralateral superior colliculus. It would be interesting knowing more about contralateral corticotectal projections to compare with corticobulbar and corticospinal projections.

4.- The major finding of this manuscript is that the density of corticotectal projections increases from SMA and PM to M1. Interestingly, in a recent paper the authors showed a similar gradient for corticobulbar projections originated in SMA, PM, and M1. In contrast, the density of corticospinal projections shows the opposite trend being higher in M1. In summary, corticobulbar and corticotectal projections from SMA and PM are denser than from M1 but corticospinal projections from M1 are denser.

Interestingly, corticocortical connections also vary along the SMA-PM-M1 trend. For instance, corticocortical connections in SMA and PM are more widespread than in M1 (Morecraft et al. Cytoarchitecture and cortical connections of the anterior cingulate and adjacent somatomotor fields in the rhesus monkey, Brain Res Bull). The architecture of SMA-PM-M1 areas also shows a gradient of progressive differentiation in parallel with functional specialization (Barbas H, García-Cabezas MÁ. Motor cortex layer 4: less is more, Trends in Neuroscience). The authors could take into account for the discussion the relation between the gradients of cortical architecture and the gradients of corticocortical, corticospinal, corticobulbar, and corticotectal projections.

Minor points:
1.- The running title is not accurate.
2.- Can the authors specify the species of the monkeys?
3.- Can the authors tell the molecular weight of BDA?

Reviewer: 2 (Elena Borra, University of Parma, Italy)

Comments to the Author
In this manuscript, Fregosi and Rouiller analyze the distribution of labeled terminals in the superior colliculus

(SC) after injections of anterograde tracers in the primary motor (M1), premotor (PM), and supplementary motor (SMA) areas. The author's hypothesis, based on the available data in the literature, is that PM areas display more cortico-tectal projections with respect to areas M1 and SMA. The authors carried out a quantitative assessment of the number of terminals, which was possible thanks to the low density of the terminals in the labeled SC territories. The results showed that the number of labeled terminals in the different layers of the SC (mainly intermediate and deep layers) is relatively high after injections in the PM and SMA, and relatively low after injections in the arm/hand region of M1. The number of labeled terminals was normalized with respect to the number of labeled corticospinal axons. This normalization procedure has, of course, "pros and cons" that have been discussed by the authors in another recent report (Fregosi et al., 2017). How these projections could contribute to motor control is briefly discussed.
This study confirms previous studies and provides further details on the descending projections from the various agranular frontal areas.

I have only one request of clarification and one suggestion.

Page 7, Results. The authors should note that the total number of terminals observed in the two cases of injections in SMA is quite different (Corrected number of boutons: 1053 vs 6513). In both cases the hand representation of SMA was injected, based on ICMS. However, is it possible that this difference is due to differences in the location of the injection sites? Is it possible that there is an involvement of the face or hindlimb region in the two cases?

After injections in PM and SMA, the labeling involved mainly the deep and the intermediate layers (laterally) along the whole rostrocaudal extent of the SC. After injections in M1, the labeling was located mainly in the deep layer in the rostral part of the SC. I suggest to briefly describe the anatomo-functional organization of the SC, to provide further elements for hypothesizing the possible functional role of these connections.

Reviewer: 3 (Richard Dum, University of Pittsburgh, USA)

Comments to the Author
General Comments: This paper examines the from the PMd/PMv, M1 and SMA to the superior colliculus. The authors quantitatively evaluate the number of boutons in each layer of the superior colliculus after separate injections of BDA into the 3 cortical motor areas in the frontal lobe. The authors are quite experienced in the technique and have notable publications in neuroanatomy. This study builds nicely on prior work with the same animals (plus a couple more) to produce a more complete picture of descending projections from frontal lobe motor areas. In particular, these results demonstrate a more widespread origin of corticotectal projections than previously reported. They found corticotectal projections from the SMA that were comparable to those originating from the PMv and PMd. Prior injections into the tectum (Fries, 1984, 1984) failed to observe such projections.

Major Comments:

1. The Introduction is difficult to understand. How does this assist in the interpretation of the results? More direct sentences with one idea per sentence would increase the clarity. Please break up long sentences . What is the point of the first paragraph? It seems like a digression that is only slightly used in the Discussion.

2. Is normalization by CST number really valid and does it enhance the interpretation of the results? Although in principle it seems like a good idea to normalize the bouton counts by some measurable number, there are multiple problems with CS normalization. 1) Fries (1984, 1985) reports that cortico-tectal projections only originate from limited portions of the PMd, PMv and M1. If the injection site is larger than the site of origin, then the normalization will falsely decrease the number of boutons because the CS count will be too high. Is there any way to control for this possibility?. However, Fries's data is not ideal since his injection sites in the superior colliculus often failed to cover the deepest layer where the present study finds the heaviest terminations. 2) The SMA, PMd and especially the PMv have lower densities of CS neurons than does M1 sulcus. This disparity will tend to elevate normalized PMd/PMv and SMA bouton numbers relative to M1 bouton numbers. That is, for the same area of injection site, PMd/PMv/SMA will have fewer CS neurons than the same sized injection site in M1. How does introducing another unknowable variable into the calculation produce a valid normalization?

3. Several issues regarding the tectospinal pathway and corticotectal pathways were omitted from the Discussion. How important is a cortical to superior colliculus to spinal cord pathway compared to the corticospinal system given the large disparity in magnitudes of these two pathways? (See Nudo RJ, Masterton RB. Descending pathways to the spinal cord: II. Quantitative study of the tectospinal tract in 23 mammals. J Comp Neurol. 1989 Aug 1;286(1):96-119.) No mention was made of the fact that Tokuno et (1995) found almost all of the M1 orofacial projections to the superior colliculus were located in the intermediate layer whereas in the present study, most of the M1 tectal projections were found in the deep layer of the SC. Any thoughts on this rather striking difference? Fries (1984, 1985) data is consistent with

the cortico-tectal projection from M1 and PMd to be from neck regions of M1, just lateral to the hand representation. Is there any evidence that the pattern of terminations in the SC is localized to regions that control neck coordination?

Specific Comments:

Abstract: No real conclusion or significance statement is included. What is the importance of this study?

It would be helpful to see the reconstruction of the SMA injection for animal Mk-Bs.

Please use same monkey numbers as Fregosi et 2017. This would facilitate comparison of the data between manuscripts.

Last paragraph of Methods. A correction for the number of sections sampled is rather straightforward and does not need more than a sentence or two at most. Besides, it is detailed in Table 1.

The Discussion would benefit from some more in depth examination of the potential functional roles for this pathway. Is it specialized for a specific role in head/neck- eye coordination or does it have just a non-specific origin throughout these 3 motor regions?

Table 1: intersection interval, not 'intersections interval'

P6- Please don't begin the Results with someone else's results. Plainly state what you found and then indicate that it confirms and extends prior results.

Fig. 5- Please remove panel A- Normalized numbers of boutons. The numbers in this histogram are not comparable since the frequency of section sampling is different. Panel B- As indicated above, this histogram should only be adjusted for section sampling frequency since normalization by CST number is problematic.

Fig. 6- This figure is difficult to look at and to compare all the different animals. Perhaps 3 histograms, one for each area injected, would be better. The 3 animals could then be 3 adjacent histogram columns and allow instant comparison with PMd, M1 and SMA.

Authors' Response 08 August 2017

Dear Editors,

Many thanks for your e-mail as well as your comments and those of the reviewers. They were all highly useful to improve our work. Please find below our replies to each comment, in red characters of your original e-mail and how the manuscript was revised accordingly.

Dear Prof. Rouiller,

Your manuscript was reviewed by three external reviewers as well as by the Section Editor, Dr. Helen Barbas, and ourselves.

The reviews collectively indicate that your experiments generated new and important information. However, there are several substantial issues that need to be clarified/resolved before we can consider your manuscript further for publication in EJN.

As you can see, the Reviewers appreciated your findings and their significance. They also provided a list of points to address in order to help focus and provide a functional context of your findings for a broad audience. One of the issues arising pertains to the normalization of the data, which is not adequately explained or justified and is subject to pitfalls. A brief significance statement in the abstract is needed. Discussion of your findings in the context of both the origin of the pathways in the cortex and their termination within different layers of the superior colliculus and their functional specialization will help point to the overall significance of the findings. In this context, old physiological studies (Abrahams and Rose, 1975) on converging responses from muscles that move the eyes and the neck in the superior colliculus of cats may be relevant. Please be consistent in the naming of your cases to allow cross referencing in your studies. It is necessary to break up long sentences for clarity and go over the manuscript carefully to correct grammatical and orthographic errors. Finally, please ensure that the reference list and in-text citations adhere to EJN guidelines.

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Based on these comments, changes have been made accordingly (see below replies to each of the 3 reviewers). In particular, the functional significance of the results has been expanded in the discussion, including a brief final statement in the abstract. The work of Abrahams and Rose (1975) has been introduced in the revised version. The naming of our cases has been modified, as requested.
The revised manuscript has been checked for language by native English speaking collaborators (see acknowledgements).

If you are able to respond fully to the points raised, we would be pleased to receive a revision of your paper within 12 weeks.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

First of all, we warmly thank the 3 reviewers (and the editors) for their constructive and highly pertinent comments in order to improve our work. The revised manuscript was modified according to the comments, as described specifically below in the text of each reviewer.

However, the “normalization” issue deserves a general comment. It is a controversial approach, as we have seen that with reviewers in our recent paper (Fregosi et al., 2017), with the present reviewers, as well as with most colleagues to whom the data were presented. In short, some reviewers/colleagues agree/support the normalization approach as an additional assessment to the raw data whereas others have concerns. Both opinions have their pros and cons (as outlined in Fregosi et al., 2017) and in the revised version of the discussion of the present manuscript (relaying the concern of Reviewer 3).

With that respect, one may consider 3 scenarios:
a) The raw data do not show any difference between the cortical areas of origin, but the normalization data do. In such a case, concerns about conclusions based on normalized data only are fully legitimate. This is fortunately not the case here.
b) The raw data show a difference between the cortical areas of origin, but not the normalized data. In such a case, variability among injections sites may be a concern. Fortunately, this is not the case here.
c) Both the raw data and the normalized data go clearly in the same direction, as it is the case in the present study. In that case, the normalized data should be considered as an additional approach to the raw data. This is what happens in the present study and therefore the revised manuscript was modified accordingly: more weight on the raw data (presented first) and normalized data reported as a complement.

Reviewer: 1
Comments to the Author
The paper “Ipsilateral corticotectal projections from the primary, premotor and supplementary motor cortical areas in adult macaque monkeys: a quantitative anterograde tracing study” traces coticotectal projections injecting the anterograde tracer BDA in the cortex of 9 monkeys. Recently, the authors have described corticobulbar projections using 7 of those monkeys.

The authors find a density gradient of corticotectal projections across motor areas. Corticotectal projections originated in premotor (PM) and supplementary motor (SMA) areas are denser than corticotectal projections originated in the primary motor area (M1).

The experiments are well designed and the findings are of interest for a general audience of neuroscientists. Here are some suggestions to improve the manuscript.

Major comments:
1.- The authors do not use unbiased stereological methods for the analysis of bouton density in corticotectal projections. In the methods they state that:

"...it was possible to visualize and chart every individual axonal bouton, en passant or terminal. In such a case, stereological techniques do not apply (e.g. Lavenex at al., 2000; Geuna 2000; Benes and Lange 2001)."

The fact that the entire population of boutons can be visualized does not exclude the use of unbiased stereological methods. Actually, if some of the labeled boutons could not be visualized then stereology could not be applied because not every bouton would have the same chances to be counted and the estimation would be biased.

The strength of stereology is that the population of particles (boutons) in the region of interest (superior colliculus) could be estimated with a known error. Even more, the number of sections analyzed and the interval between them would be taken into account to calculate the error and no further correction would be needed. The authors' choice for exhaustive plotting instead of stereology is legitimate and enough to show trends in corticotectal projections across motor areas, but stereological methods are the gold standard and they are not excluded because of the visualization of boutons.

OK, the point is well taken. We agree that stereology is the gold standard. Unfortunately, we do not have access to the stereology function in our (old) Neurolucida equipment. Furthermore, for the present report, it made sense to use the very same method as in our previous study on the corticobulbar (corticoreticular) projection (Fregosi et al., 2017).

2.- The authors normalize the number of BDA labelled axonal boutons by the corresponding number of BDA labelled corticospinal axons in the same animal at the level of the pyramid, multiplied by 1000. This part of the methods, which is key for the interpretation of the data could be better explained. Why multiply by 1000? In how many sections did the authors counted BDA labeled axons?

Dividing the number of boutons by the number of CS labelled axons yield low number (ranging from 0 to 6, smaller than 1 in some cases), with several decimals. In order to obtain numbers without decimals, we arbitrarily multiplied by 1000, though it corresponds to the order of magnitude of the number of CS axons obtained across our monkey population (range 703 to 3195: see Table 1).

The number of CS axons was counted in one section above the pyramidal decussation in most cases. However, in previous studies (e.g. Rouiller et al., 1996, including the monkeys M1-3 and SMA-1 of the present sudy), we had performed repeated counts of CS axons in adjacent sections and we had obtained very comparable numbers. Based on this control procedure, counts were made on one section later on.

3.- The authors analyzed ipsilateral corticotectal projections but in the discussion they mention that few if any axonal terminal boutons were seen in the contralateral superior colliculus. It would be interesting knowing more about contralateral corticotectal projections to compare with corticobulbar and corticospinal projections.

The contralateral SC has been scanned during the revision procedure, confirming the paucity of corticotectal axonal boutons. A new paragraph has been inserted in the results to report on those observations (page 8). The discussion was also modified accordingly (pages 9-10), including the insertion of a comparison in terms of laterality with the corticobulbar and corticospinal projections.

4.- The major finding of this manuscript is that the density of corticotectal projections increases from SMA and PM to M1. Interestingly, in a recent paper the authors showed a similar gradient for corticobulbar projections originated in SMA, PM, and M1. In contrast, the density of corticospinal projections shows the opposite trend being higher in M1. In summary, corticobulbar and corticotectal projections from SMA and PM are denser than from M1 but corticospinal projections from M1 are denser.

Interestingly, corticocortical connections also vary along the SMA-PM-M1 trend. For instance, corticocortical connections in SMA and PM are more widespread than in M1 (Morecraft et al. Cytoarchitecture and cortical connections of the anterior cingulate and adjacent somatomotor fields in the rhesus monkey, Brain Res Bull). The architecture of SMA-PM-M1 areas also shows a gradient of progressive differentiation in parallel with functional specialization (Barbas H, García-Cabezas MÁ. Motor cortex layer 4: less is more, Trends in Neuroscience). The authors could take into account for the discussion the relation between the gradients of cortical architecture and the gradients of

corticocortical, corticospinal, corticobulbar, and corticotectal projections.

In the revised discussion, a paragraph has been added, emphasizing this notion of gradient, extended to corticortical connections and laminar architecture (page 11). The corresponding references have been inserted in the revised version.

Minor points:
1.- The running title is not accurate.
Modified accordingly.

2.- Can the authors specify the species of the monkeys?
The species were indicated in Table 1. However, in the revised version, this information has been added in the text itself (first paragraph of methods), as proposed by the reviewer.

3.- Can the authors tell the molecular weight of BDA?
MW=10'000: inserted in the revised version.

Reviewer: 2
Comments to the Author
In this manuscript, Fregosi and Rouiller analyze the distribution of labeled terminals in the superior colliculus (SC) after injections of anterograde tracers in the primary motor (M1), premotor (PM), and supplementary motor (SMA) areas. The author's hypothesis, based on the available data in the literature, is that PM areas display more cortico-tectal projections with respect to areas M1 and SMA. The authors carried out a quantitative assessment of the number of terminals, which was possible thanks to the low density of the terminals in the labeled SC territories. The results showed that the number of labeled terminals in the different layers of the SC (mainly intermediate and deep layers) is relatively high after injections in the PM and SMA, and relatively low after injections in the arm/hand region of M1. The number of labeled terminals was normalized with respect to the number of labeled corticospinal axons. This normalization procedure has, of course, "pros and cons" that have been discussed by the authors in another recent report (Fregosi et al., 2017). How these projections could contribute to motor control is briefly discussed.

This study confirms previous studies and provides further details on the descending projections from the various agranular frontal areas.

I have only one request of clarification and one suggestion.

Page 7, Results. The authors should note that the total number of terminals observed in the two cases of injections in SMA is quite different (Corrected number of boutons: 1053 vs 6513). In both cases the hand representation of SMA was injected, based on ICMS. However, is it possible that this difference is due to differences in the location of the injection sites? Is it possible that there is an involvement of the face or hindlimb region in the two cases?

Correct, the injection site has a larger rostrocaudal extent in SMA-2 than SMA-1, in line with the number of corticotectal boutons. Then, a spread of BDA to the head representation may explain the larger number of boutons. This issue has been mentioned in the revised version of the manuscript in the discussion (page 10).

After injections in PM and SMA, the labeling involved mainly the deep and the intermediate layers (laterally) along the whole rostrocaudal extent of the SC. After injections in M1, the labeling was located mainly in the deep layer in the rostral part of the SC. I suggest to briefly describe the anatomo-functional organization of the SC, to provide further elements for hypothesizing the possible functional role of these connections.

Modified accordingly, in the introduction section (pages 3-4) and in the discussion (several places, pages 8-12).

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Reviewer: 3
Comments to the Author
General Comments: This paper examines the projection from the PMd/PMv, M1 and SMA to the superior colliculus. The authors quantitatively evaluate the number of boutons in each layer of the superior colliculus after separate injections of BDA into the 3 cortical motor areas in the frontal lobe. The authors are quite experienced in the technique and have notable publications in neuroanatomy. This study builds nicely on prior work with the same animals (plus a couple more) to produce a more complete picture of descending projections from frontal lobe motor areas. In particular, these results demonstrate a more widespread origin of corticotectal projections than previously reported. They found corticotectal projections from the SMA that were comparable to those originating from the PMv and PMd. Prior injections into the tectum (Fries, 1984, 1984) failed to observe such projections.

Major Comments:
1. The Introduction is difficult to understand. How does this assist in the interpretation of the results? More direct sentences with one idea per sentence would increase the clarity. Please break up long sentences.

Modified accordingly.

What is the point of the first paragraph? It seems like a digression that is only slightly used in the Discussion.
Modified accordingly, first paragraph of the introduction of the initial version has been removed in the revised version.

2. Is normalization by CST number really valid and does it enhance the interpretation of the results? Although in principle it seems like a good idea to normalize the bouton counts by some measurable number, there are multiple problems with CS normalization. 1) Fries (1984, 1985) reports that corticotectal projections only originate from limited portions of the PMd, PMv and M1. If the injection site is larger than the site of origin, then the normalization will falsely decrease the number of boutons because the CS count will be too high. Is there any way to control for this possibility?. However, Fries's data is not ideal since his injection sites in the superior colliculus often failed to cover the deepest layer where the present study finds the heaviest terminations. 2) The SMA, PMd and especially the PMv have lower densities of CS neurons than does M1 sulcus. This disparity will tend to elevate normalized PMd/PMv and SMA bouton numbers relative to M1 bouton numbers. That is, for the same area of injection site, PMd/PMv/SMA will have fewer CS neurons than the same sized injection site in M1. How does introducing another unknowable variable into the calculation produce a valid normalization?

See general comment above (on top of the reply). In line with the reviewer's concern, the normalized data were not used to enhance the interpretation of the results (as this was the case in the initial version), but rather used as an additional (secondary) supporting argument. As a consequence, in the Figure 5, the raw data were moved to panel A, whereas the normalized data were reduced to panel B, coming in a second and more limited step of the results' description. The concerns of the reviewer with respect to the normalization were inserted in the revised version of the manuscript (page 9). The issue of location of injection sites by Fries has also been inserted in the revised version of the manuscript, as it may explain why Fries did not report a corticotectal projection from SMA (page 9).

3. Several issues regarding the tectospinal pathway and corticotectal pathways were omitted from the Discussion. How important is a cortical to superior colliculus to spinal cord pathway compared to the corticospinal system given the large disparity in magnitudes of these two pathways? (See Nudo RJ, Masterton RB. Descending pathways to the spinal cord: II. Quantitative study of the tectospinal tract in 23 mammals. J Comp Neurol. 1989 Aug 1;286(1):96-119.) No mention was made of the fact that Tokuno et (1995) found almost all of the M1 orofacial projections to the superior colliculus were located in the intermediate layer whereas in the present study, most of the M1 tectal projections were found in the deep layer of the SC. Any thoughts on this rather striking difference? Fries (1984, 1985) data is consistent with the cortico-tectal projection from M1 and PMd to be from neck regions of M1, just lateral to the hand representation. Is there any evidence that the pattern of terminations in the SC is localized to regions that control neck coordination?

The issue of the relatively minor importance of the tectospinal projection, based on an evolutionary perspective as reported by Nudo and Masterton (1989) has been introduced in the revised discussion (in

its last paragraph on page 12).

The layer difference with the study of Tokuno et al. (1995) has been mentioned now in the revised version of the discussion (page 10), suggesting different roles for the SC in the control of movements depending on the body territory (face versus arm/hand).

Specific Comments:
Abstract: No real conclusion or significance statement is included. What is the importance of this study?
A conclusion and functional significance statement has been added at the end of the abstract (within the 250 words limit).

It would be helpful to see the reconstruction of the SMA injection for animal Mk-Bs.
Introduced in the revised version of Fig. 4.

Please use same monkey numbers as Fregosi et 2017. This would facilitate comparison of the data between manuscripts.
Modified accordingly in the text, in Table 1 and in the Figures.

Last paragraph of Methods. A correction for the number of sections sampled is rather straightforward and does not need more than a sentence or two at most. Besides, it is detailed in Table 1.
Modified accordingly (reduced to two sentences).

The Discussion would benefit from some more in depth examination of the potential functional roles for this pathway. Is it specialized for a specific role in head/neck- eye coordination or does it have just a non-specific origin throughout these 3 motor regions?
Modified accordingly in the revised discussion.

Table 1: intersection interval, not 'intersections interval'
Modified accordingly.

P6- Please don't begin the Results with someone else's results. Plainly state what you found and then indicate that it confirms and extends prior results.
Modified accordingly (the reference to prior results is restricted to the discussion).

Fig. 5- Please remove panel A- Normalized numbers of boutons. The numbers in this histogram are not comparable since the frequency of section sampling is different. Panel B- As indicated above, this histogram should only be adjusted for section sampling frequency since normalization by CST number is problematic.
See general comment on "normalization" above. As proposed by the reviewer, the initial panel A (normalized data in the initial Fig. 5) has been removed and replaced by the raw data, including though the correction related to intersection intervals (see Table 1). In other words, in the initial version, normalization preceded the intersection interval correction. In the revised version, the intersection interval logically comes first. Then, as explained in the general comment, the panel B offers the normalization data.

Fig. 6- This figure is difficult to look at and to compare all the different animals. Perhaps 3 histograms, one for each area injected, would be better. The 3 animals could then be 3 adjacent histogram columns and allow instant comparison with PMd, M1 and SMA.
Figure 6 has been modified accordingly.

2nd Editorial Decision 21 August 2017

Dear Prof. Rouiller,

Your revised manuscript was re-evaluated by external reviewers as well as by the Section Editor, Dr. Helen Barbas and ourselves. We are pleased to inform you that we expect that it will be acceptable for publication in EJN following further revision and possible re-review.

As you can see, the Reviewers acknowledged your effort in addressing the points raised in their reviews. One Reviewer provided a series of points to help stress the essential findings and their

significance and has generously re-worded some passages for clarity. Reviewer 2 pointed to a few minor edits, but the main point pertains to the quantitative methods used to analyze data. The journal does not require that you use stereological methods, but it is still necessary not to misrepresent the method. Please delete the incorrect statement, “In such a case, stereological techniques do not apply”. People can and do use stereology even when sampling is exhaustive. Please also provide a graphical abstract.

If you are able to respond fully to the points raised, we shall be pleased to receive a revision of your paper within 30 days.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 3 (Richard Dum, University of Pittsburgh, USA)

Comments to the Author
General Comments: The authors have made considerable corrections to the prior manuscript. My only concern is with the clarity and conciseness of the writing. I have attempted to provide some guidance in that area. I think that increasing the clarity is important because the study has some nice results that sometimes tend to be lost.

Specific Comments:

I took the liberty of rewriting some paragraphs. This was actually easier than trying to indicate all the subtle English usage and logical improvements that could be made. Another issue is the complexity of sentence structure used in the manuscript. Fewer words and more direct statements result in a higher impact. Revisions were rather easy because the information was mostly well laid out. Feel free to use these words as you see fit but in any case, try to understand the logic behind the changes

Abstract: Suggested rewrite for clarity. I removed the direct corticospinal projections as they only originate from M1 and seem to be a digression from the main point.

The corticotectal projection from cortical motor areas is one of several descending pathways involved in the indirect control of spinal motoneurons. In non-human primates, previous studies reported that cortical projections to the superior colliculus originated from the premotor cortex and the primary motor cortex, whereas no projections originated from the supplementary motor area. The aim of the present study was to investigate and compare the properties of corticotectal projections originating from these three cortical motor areas in intact adult macaques (n=9). The anterograde tracer BDA was injected into one of these cortical areas in each animal. Individual axonal boutons, both en passant and terminaux, were charted and counted in the different layers of the ipsilateral superior colliculus. The data confirmed the presence of strong corticotectal projections from the premotor cortex. A new observation was
that dense corticotectal projections were also found to originate from the supplementary motor area (its proper division). The corticotectal projection from the primary motor cortex was quantitatively less dense than that from either the premotor or supplementary motor areas. The corticotectal projection from each motor area was directed mainly to the deep layer of the superior colliculus, although its intermediate layer was also a consistent target of fairly dense terminations. The dense corticotectal projections from non-primary motor areas are in position to influence the preparation and planning of voluntary movements.

Results- first red paragraph, p16
Note that I tried to avoid the term “density” in my rewrite. Density refers to number per unit area which was not determined (another form of normalization). Only total numbers of boutons is presented.

EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Replacement for two middle paragraphs on p16.
The numbers of axonal boutons counted in the SC for each motor area are compared in Table 1 and Fig. 5. The raw numbers of axonal boutons were corrected according to the interval of section sampling across monkeys (see Methods). The corrected numbers of axonal boutons in SC were highest for the PM, somewhat lower for the SMA-proper and much lower for M1 (Fig. 5A).
To further compare the density of the corticotectal projections originating from PM,
M1 or SMA-proper, the total corrected numbers of axonal boutons in SC were normalized based on the number of CS axons obtained for the same BDA injection (see Methods). After normalization, the conclusions based on the corrected numbers of axonal boutons (Fig. 5A) were largely validated (Fig. 5B). The corticotectal projection from M1 is considerably
weaker than those originating from the two premotor areas (PM and SMA-proper).
Furthermore, the normalized data along with the inter-individual variability among animals suggest that the strength of the corticotectal projections from the PM and SMA (Fig. 5B) are comparable.

P16- last sentence. Please avoid the term “significant” unless referring to statistical tests. Suggest- In most cases, there was also a substantial projection terminating in SCint. This projection, however, was quite variable from one animal to the next, especially for the projections originating from M1 and to a lesser extent for those from PM.

P17- last para of Results
The corticotectal projections from PM, SMA-proper or M1 appeared to be mostly restricted to the ipsilateral SC. Although of few BDA labeled axons were found in the SC contralateral to the injected hemisphere, most of these axons were passing stem axons that did not emit boutons, en passant or terminaux. However, rare axonal boutons were observed in SCint and SCdeep contralaterally. When present, these corticotectal axonal boutons in the contralateral SC were located in regions that mirrored the main projection territories in the ipsilateral SC. The percentage of the total number of axonal boutons observed in the contralateral SC ranged from 0.0% to 1.6% in all but one monkey (SMA-1) where the contralateral percentage was 4.8%.

Discussion
An alternative view of your results. Try not to dissipate the importance of your results with statements that the hypothesis was not confirmed or complex comparisons with prior work. Emphasize the importance of what you found and how it confirms and extends prior work.

‘The present analyses demonstrate that the PM, SMA-proper and M1 have significant projections to the ipsilateral superior colliculus. The major new finding is that the SMA-proper gives rise to a significant corticotectal projection (Fig. 4; Table 1). This SMA projection is similar in strength to that of the PM and considerably stronger than that originating from M1 (Fig. 5). The presence of corticotectal projections from the SMA was unexpected since a prior retrograde tracing study failed to observe labeling in the SMA after HRP injections into the superior colliculus (Fries 1984, 1985). Our observations are consistent with and extend prior studies (Borra et al., 2010, 2014; Distler and Hoffmann 2015) that examined corticotectal projections from PM. Our quantitative analysis, like those prior qualitative studies, found that SCint and SCdeep are the major targets of the corticotectal projection originating from PM whereas there was only a very weak corticotectal projection originating from motor cortical areas to SCsup. Overall, our observations demonstrate that corticotectal projections originate from more widespread regions of the motor areas in the frontal lobe than previously recognized and that these projections target the deep layers of the superior colliculus.
The failure of prior studies (Fries 1984, 1985) to detect projections from the SMA to the superior colliculus may result from two factors. First, Fries used HRP as his retrograde tracer and HRP is less sensitive than cholera toxin used in the present study (Ref?). Second, most of his HRP injections in superior collicus did not include the deepest layers of the SC where we found the heaviest projections from the SMA (Figs. 4, 6). Thus, his injections sites may have largely avoided the sites with the densest SMA terminations.’

“The present study, like recent reports (Borra et al., 2010, 2014; Distler and Hoffmann 2015) took advantage of the BDA tracing technique which allows visualization of synaptic boutons within axon terminal fields. Furthermore, we could quantify the numbers of corticotectal boutons due to the spatially limited and relatively sparse terminal fields. Possible limitations of this quantitative analysis reside in variations of BDA injection sites, uptake and transport as well as the different sampling

intervals of histological sections across monkeys. We attempted to attenuate these possible limitations by correcting for section sampling intervals and by normalizing the data based on CS axon counts (Fig. 5; see Fregosi et al. 2017 for a more complete discussion of the normalization procedure). One limitation of our normalization procedure is that the corticotectal projections originate from limited territories of PM or M1 (Fries 1984, 1985) whereas the BDA injection may spread beyond the cortical territories projecting to the SC. This would result in an elevated CS axon count that reflected CS axons originating both within and outside of the SC projection territory. As a consequence, the normalized number of boutons in SC would be decreased. In addition, the PM and SMA tend to have a slightly lower density of CS axons than M1 (Dum and Strick, 1991), which would increase the normalized number of boutons from PM and SMA as compared to M1. Despite these potential limitations, the trend in the normalized number of boutons among the motor areas is the same as the trend in the raw data (Fig. 6). Therefore, normalized data support the conclusion derived form the raw data, namely that the PM and SMA have stronger projections to the SC than does M1."

P19- next to last paragraph (in red) does not seem to add anything to the Discussion. I suggest removal.

P20- suggest- "However, the CS component from M1 outnumbers that coming from the PM or SMA (Dum and Strick, 1991, 1996;p Rouiller et al, 1996), whereas the projection strength is reversed (denser projections from the PM and SMA than from M1) for the corticotectal (present study) and corticobulbar projections (Fregosi et al., 2017)." .

P20, line 18- See also Markov et al. Cerebral Cortex 2014 for an expanded view of this issue.

P21- From an evolutionary point of view (Nudo and Masterton, 1989), the tectospinal projection in the non-human primate represents quantitatively a minor descending projection in comparison with the more massive corticospinal and rubrospinal projections. The size of the tectospinal projection does not correlate with good vision, manual dexterity or hand-eye coordination (Nudo and Masterton, 1989). As a consequence, the moderate number of corticotectal axon terminals observed in the present study suggest that these projections may contribute to a motor based regulation of intrinsic neuronal circuits in the deeper layers of the SC. One may speculate that the motor corticotectal projection provides the SC with an efferent copy of the voluntary movement programs. This efference copy could be used locally for sensorimotor integration in the SC rather than as an indirect route from the cerebral cortex to spinal motoneurons via the tectum. For M1, such motor influence on the SC appears to be focused on the orofacial representation (Tokuno et al., 1995), whereas it may involve more extensive body part representations from the PM and SMA (Borra et al., 2010, 2014; Distler and Hoffmann 2015; present study).

Fig. 6, Table 1- I think it would be better to label SMA-3 as preSMA so as to not cause confusion. A cursory examination of Fig. 6 might lead one to conclude that some SMA-proper injections do not result in corticotectal projections.

Reviewer: 1 (Miguel Angel García-Cabezas, Boston University, USA)

Comments to the Author
EJN-2017-06-24621.R1
Ipsilateral corticotectal projections from the primary, premotor and supplementary motor cortical areas in adult macaque monkeys: a quantitative anterograde tracing study

The authors have addressed all the points raised by the editors and the reviewers and the manuscript has been improved, but this reviewer still has some concerns with the description of the quantification method.

1.- The authors still state in the methods that "…it was possible to visualize and chart every individual axonal bouton, en passant or terminal. In such a case, stereological techniques do not apply (e.g. Lavenex at al., 2000; Geuna 2000; Benes and Lange 2001)."

I must insist in that this statement is incorrect. A prerequisite for stereology is visualization of every individual element of the population that will be quantified in the region of interest, otherwise not

every element (labeled boutons) in the region of interest (tectum) would have the same chances to be counted and the estimation would be biased. Stereological techniques do apply in this case.

The authors chose a different quantification method. Instead of stereology they used exhaustive plotting. This choice is legitimate and served well enough to show differences in corticotectal projections. The authors could explain in the methods the reasons for having used exhaustive plotting instead of sterology; for instance, it allows comparison with a previous paper on corticoreticular projections using the same cases and the same quantification method. This reviewer respect the choice of the authors regarding the quantification method, but the statement on the applicability of stereology is untenable.

2.- Regarding the "normalization" issue, this reviewer finds very interesting the reasoning given by the authors in the reviews, both in the general comment and in the answer to point 2. Why not adding all these comments to the methods and the discussion? That would allow readers of this paper to exercise their critical judgement on the advantages and disadvantages of the different quantification methods that neuroanatomists have at hand.

3.- In Table 1 and throughout the text the authors should write numbers consistently: 10000 or 10'000 should be 10,000.

4.- In Figure 2, "BDA injection in PM" could be "BDA injection in PMd/PMv".

Authors' Response 25 August 2017

Dear Editors,
Many thanks for your e-mail as well as your comments and those of the two reviewers. They were all pertinent and very useful to improve our work.

Please find below our replies to each comment, in red characters within your original e-mail and how the manuscript was revised accordingly.

Dear Prof. Rouiller,

Your revised manuscript was re-evaluated by external reviewers as well as by the Section Editor, Dr. Helen Barbas and ourselves. We are pleased to inform you that we expect that it will be acceptable for publication in EJN following further revision and possible re-review.

As you can see, the Reviewers acknowledged your effort in addressing the points raised in their reviews. One Reviewer provided a series of points to help stress the essential findings and their significance and has generously re-worded some passages for clarity. Reviewer 2 pointed to a few minor edits, but the main point pertains to the quantitative methods used to analyze data. The journal does not require that you use stereological methods, but it is still necessary not to misrepresent the method. Please delete the incorrect statement, "In such a case, stereological techniques do not apply". People can and do use stereology even when sampling is exhaustive. Please also provide a graphical abstract.
The re-revised version has been modified accordingly (see below for detail) and a graphical abstract was introduced.

If you are able to respond fully to the points raised, we shall be pleased to receive a revision of your paper within 30 days.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Reviewer: 3

Comments to the Author
General Comments: The authors have made considerable corrections to the prior manuscript. My only concern is with the clarity and conciseness of the writing. I have attempted to provide some guidance in that area. I think that increasing the clarity is important because the study has some nice results that sometimes tend to be lost.

Specific Comments:

I took the liberty of rewriting some paragraphs. This was actually easier than trying to indicate all the subtle English usage and logical improvements that could be made. Another issue is the complexity of sentence structure used in the manuscript. Fewer words and more direct statements result in a higher impact. Revisions were rather easy because the information was mostly well laid out. Feel free to use these words as you see fit but in any case, try to understand the logic behind the changes

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Thank you. Abstract replaced by the version proposed by the reviewer. Nevertheless, in the proposed version, we replace “dense” by “strong” (see next reviewer’s comment).

Results- first red paragraph, p16
Note that I tried to avoid the term “density” in my rewrite. Density refers to number per unit area which was not determined (another form of normalization). Only total numbers of boutons is presented.
In the re-revised version, the term “density” of projection was avoided in the entire manuscript, replaced by “strength” of projection.

Replacement for two middle paragraphs on p16.

The numbers of axonal boutons counted in the SC for each motor area are compared in Table 1 and Fig. 5. The raw numbers of axonal boutons were corrected according to the interval of section sampling across monkeys (see Methods). The corrected numbers of axonal boutons in SC were highest for the PM, somewhat lower for the SMA-proper and much lower for M1 (Fig. 5A).

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Furthermore, the normalized data along with the inter-individual variability among animals suggest that the strength of the corticotectal projections from the PM and SMA (Fig. 5B) are comparable.
Thank you. Modified accordingly (see page 7). The word “density” was however replaced by “strength”.

P16- last sentence. Please avoid the term “significant” unless referring to statistical tests. Suggest- In most cases, there was also a substantial projection terminating in SCint. This projection, however, was quite variable from one animal to the next, especially for the projections originating from M1 and to a lesser extent for those from PM.
Thank you, modified accordingly (see page 7).

P17- last para of Results
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Thank you, modified accordingly (see page 8).

Discussion
An alternative view of your results. Try not to dissipate the importance of your results with statements that the hypothesis was not confirmed or complex comparisons with prior work. Emphasize the importance of what you found and how it confirms and extends prior work.

‘The present analyses demonstrate that the PM, SMA-proper and M1 have significant projections to the ipsilateral superior colliculus. The major new finding is that the SMA-proper gives rise to a significant corticotectal projection (Fig. 4; Table 1). This SMA projection is similar in strength to that of the PM and considerably stronger than that originating from M1 (Fig. 5). The presence of corticotectal projections from the SMA was unexpected since a prior retrograde tracing study failed to observe labeling in the SMA after HRP injections into the superior colliculus (Fries 1984, 1985). Our observations are consistent with and extend prior studies (Borra et al., 2010, 2014; Distler and Hoffmann 2015) that examined corticotectal projections from PM. Our quantitative analysis, like those prior qualitative studies, found that SCint and SCdeep are the major targets of the corticotectal projection originating from PM whereas there was only a very weak corticotectal projection originating from motor cortical areas to SCsup. Overall, our observations demonstrate that corticotectal projections originate from more widespread regions of the motor areas in the frontal lobe than previously recognized and that these projections target the deep layers of the superior colliculus.

The failure of prior studies (Fries 1984, 1985) to detect projections from the SMA to the superior colliculus may result from two factors. First, Fries used HRP as his retrograde tracer and HRP is less sensitive than cholera toxin used in the present study (Ref?). Second, most of his HRP injections in superior colliculus did not include the deepest layers of the SC where we found the heaviest projections from the SMA (Figs. 4, 6). Thus, his injections sites may have largely avoided the sites with the densest SMA terminations.’
Thank you, modified accordingly. However, “significant” was replaced by “substantial” (see your comment above). Furthermore (pages 8-9), cholera toxin was not used in the present study (replaced by BDA).

“The present study, like recent reports (Borra et al., 2010, 2014; Distler and Hoffmann 2015) took advantage of the BDA tracing technique which allows visualization of synaptic boutons within axon terminal fields. Furthermore, we could quantify the numbers of corticotectal boutons due to the spatially limited and relatively sparse terminal fields. Possible limitations of this quantitative analysis reside in variations of BDA injection sites, uptake and transport as well as the different sampling intervals of histological sections across monkeys. We attempted to attenuate these possible limitations by correcting for section sampling intervals and by normalizing the data based on CS axon counts (Fig. 5; see Fregosi et al. 2017 for a more complete discussion of the normalization procedure). One limitation of our normalization procedure is that the corticotectal projections originate from limited territories of PM or M1 (Fries 1984, 1985) whereas the BDA injection may spread beyond the cortical territories projecting to the SC. This would result in an elevated CS axon count that reflected CS axons originating both within and outside of the SC projection territory. As a consequence, the normalized number of boutons in SC would be decreased. In addition, the PM and SMA tend to have a slightly lower density of CS axons than M1 (Dum and Strick, 1991), which would increase the normalized number of boutons from PM and SMA as compared to M1. Despite these potential limitations, the trend in the normalized number of boutons among the motor areas is the same as the trend in the raw data (Fig. 6). Therefore, normalized data support the conclusion derived from the raw data, namely that the PM and SMA have stronger projections to the SC than does M1.”

Thank you, modified accordingly (see page 9), although it was integrated with the recommendation of the reviewer 1 (his/her point 2).

P19- next to last paragraph (in red) does not seem to add anything to the Discussion. I suggest removal.
This part was requested by another reviewer in the previous revision. We propose to keep it.

P20- suggest- "However, the CS component from M1 outnumbers that coming from the PM or SMA (Dum and Strick, 1991, 1996;p Rouiller et al, 1996), whereas the projection strength is reversed (denser projections from the PM and SMA than from M1) for the corticotectal (present study) and corticobulbar projections (Fregosi et al., 2017)." .
Modified accordingly (see page 11).

P20, line 18- See also Markov et al. Cerebral Cortex 2014 for an expanded view of this issue.
Introduced in the discussion (page 11) and in the bibliography.

P21- From an evolutionary point of view (Nudo and Masterton, 1989), the tectospinal projection in the non-human primate represents quantitatively a minor descending projection in comparison with the more massive corticospinal and rubrospinal projections. The size of the tectospinal projection does not correlate with good vision, manual dexterity or hand-eye coordination (Nudo and Masterton, 1989). As a consequence, the moderate number of corticotectal axon terminals observed in the present study suggest that these projections may contribute to a motor based regulation of intrinsic neuronal circuits in the deeper layers of the SC. One may speculate that the motor corticotectal projection provides the SC with an efferent copy of the voluntary movement programs. This efference copy could be used locally for sensorimotor integration in the SC rather than as an indirect route from the cerebral cortex to spinal motoneurons via the tectum. For M1, such motor influence on the SC appears to be focused on the orofacial representation (Tokuno et al., 1995), whereas it may involve more extensive body part representations from the PM and SMA (Borra et al., 2010, 2014; Distler and Hoffmann 2015; present study).
Thank you, modified accordingly (see page 12).

Fig. 6, Table 1- I think it would be better to label SMA-3 as preSMA so as to not cause confusion. A cursory examination of Fig. 6 might lead one to conclude that some SMA-proper injections do not result in corticotectal projections.
Modified accordingly in both Table 1 and Figs. 1 and 6.

Reviewer: 1

Comments to the Author
EJN-2017-06-24621.R1
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The authors have addressed all the points raised by the editors and the reviewers and the manuscript has been improved, but this reviewer still has some concerns with the description of the quantification method.

1.- The authors still state in the methods that "...it was possible to visualize and chart every individual axonal bouton, en passant or terminal. In such a case, stereological techniques do not apply (e.g. Lavenex at al., 2000; Geuna 2000; Benes and Lange 2001)."

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The authors chose a different quantification method. Instead of stereology they used exhaustive plotting. This choice is legitimate and served well enough to show differences in corticotectal projections. The authors could explain in the methods the reasons for having used exhaustive plotting instead of sterology; for instance, it allows comparison with a previous paper on corticoreticular projections using the same cases and the same quantification method. This reviewer respects the choice of the authors regarding the quantification method, but the statement on the applicability of stereology is untenable.

The manuscript was re-revised accordingly. The untenable sentence was removed, as well as the 3 references related to it. As proposed by the reviewer, we introduced the methodological concept of exhaustive plotting, justified to allow comparison with the corticoreticular projection in the same cases, with the same quantification method (see page 5).

2.- Regarding the "normalization" issue, this reviewer finds very interesting the reasoning given by the authors in the reviews, both in the general comment and in the answer to point 2. Why not adding all these comments to the methods and the discussion? That would allow readers of this paper to exercise their critical judgement on the advantages and disadvantages of the different quantification methods that neuroanatomists have at hand.

Modified accordingly: additional issues on normalization procedure have been inserted in the re-revised manuscript in the discussion (see page 9). In the methods, we also introduced the issue of the number of sections from which the number of BDA labelled CS axons was estimated (see page 6). Similarly, the justification of the multiplication by 1000 in the normalization procedure was added in the methods (see page 6).

3.- In Table 1 and throughout the text the authors should write numbers consistently: 10000 or 10'000 should be 10,000.
All values above 1'000 were written consistently using the 1'000 convention. This may be corrected by the editorial staff if this journal uses the 1,000 convention (we did not find a policy with that respect in the authors' guidelines).

4.- In Figure 2, "BDA injection in PM" could be "BDA injection in PMd/PMv".
Figure 2 was modified accordingly.