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Neuronal connections of direct and indirect pathways for stable value memory in caudal basal ganglia

Hidetoshi Amita, Hyoung F. Kim, Mitchell Smith, Atul Gopaland Okihide Hikosaka

Review timeline:

Submission date:	6 December 2017
Editorial Decision:	8 January 2018
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Editor: Yoland Smith

1st Editorial Decision 8 January 2018

Dear Dr. Amita,

Your manuscript has been reviewed by three external reviewers as well as by the Section Editor, Dr. Yoland Smith, 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 in a revised version of the manuscript before we can consider it further for publication in EJN.

As you can see, the three reviewers found significant merit to your study and we consider that it will be an important addition to the IBAGS Special Issue. However, two of them raised some important technical and interpretative issues that must be dealt with carefully in the revised manuscript. In particular, reviewer 1 raises a series of comments that necessitate more clarity in the methods section and results description. Please, pay careful attention to each of these comments and try to address each of the points in your letter and through changes in the manuscript. Make sure all details about the quantitative approaches used are provided. Also make sure that the methods section is focused on experiments presented in this specific paper. Additional methodological information that is not relevant to studies being presented in this manuscript should be deleted. Some good suggestions are made by the reviewers about the reorganization of figures. Please, pay attention to these as you revise the manuscript.

We also noted the following points that need to be addressed
- Please provide more details for the surgical procedures (anaesthesia, peri-operative care etc.).
- Antibody dilutions used for experiment 1 immunofluorescence?
- Please provide a data statement.
- The reference list needs a careful proof-read.

Thank you for submitting your work to EJN.

Kind regards,

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

Reviews:

Reviewer: 1 Jose Lanciego (University of Navarra, Spain)

Comments to the Author
Here the authors evaluated the extent to which basal ganglia direct and indirect pathways are segregated to each other at the level of the most caudally-located basal ganglia territories such as the tail of the caudate nucleus (CDt), the caudal-dorsal-lateral part of the substantia nigra pars reticulata (cdlSNr) and caudo-ventral parts of the external division of the globus pallidus (cvGPe). To this aim, injections of anterograde tracers were achieved in the CDt to evaluate distribution of CDt efferents. Furthermore, retrograde tracers were deposited into cdlSNr and cvGPe to further assess to what extent -if any- CDt parent neurons innervating the cdlSNr and the cvGPe were different cellular populations.

1. Materials and Methods section: I have found this section particularly confusing and very difficult to read. It took me a while to understand simple things like which tracers were injected in experiments 1 and 2 and in which locations. In its present form, the narrative is very hard to understand. If I am right, the authors have used CTB555 for anterograde tract-tracing purposes, this tracer being injected into CDt (experiment 1) and in cvGPe (experiment 2). I guess this section should be rewritten entirely to facilitate an adequate and easy-going understanding.

1.1. Materials and methods, subheading "Cell-counting": "The images from confocal microscope were inspected systematically, and single labeled (CTB555, CTB488 or FB) or double labeled neurons were identified". Please indicate number of sections analyzed as well as the overall number of counted neurons labeled with each of the tracers used.

1.2. In keeping with the former comment and when dealing with neuronal counting, the Authors should explain whether all different tracers did behave the same in terms of number of neurons displaying tracing. In other words, did FB, CTB488 and CTB555 showed a similar efficacy for retrograde neuronal labeling?

1.3. Materials and methods, questions regarding "Experiment 1": For experiment 1, authors have used tissue sections available from a former study (as stated in the first paragraph of the Materials and methods section). However, the injected tracer and the place of injection (CTB555 delivered into the CDt) was only mentioned later on in the Results section (subheading "Caudate tail (CDt) directly projects to cdlSNr and cvGPe).

1.4. Although I am not fully sure, it seems that CTB555 was the Authors' main choice for anterograde tract-tracing purposes. Although I am perfectly aware that CTB is a bi-directional tracer, I guess that CTB488 should also produce anterograde labeling of fibers, in a similar fashion than CTB555.

1.5. Please note that the main aims of this study were (1) to identify cdlSNr and cvGPe territories innervated by CDt efferents and (2) to disclose the precise localization and potential co-labeling of CDt neurons innervating either cdlSNr or cvGPe. In this regard and although I also understand that the authors want to correlate these pathways with the anatomical substrates underlying long-term value coding of visual objects (i.e., stable value memory), it is evident that this manuscript is largely a neuroanatomical study with an inherent value in itself, regardless the functional counterpart. Accordingly, I would like to suggest the Authors removing all parts of the Materials and Methods sections related to Stimuli, Behavioral tasks, and Neuronal recording (i.e., rewrite everything with a narrower focus on tract-tracing experiments).

2.1. Results, subheading "tracer injections in the stable value-coding regions…". The title of this section is misleading, because the engagement of cdlSNr and cvGPe regions in stable value memory coding is taken as granted. Please change to "tracer injections in cdlSNr and cvGPe".

2.2. Results, subheading "tracer injections in the stable value-coding…", second paragraph. Here the authors quoted the following sentence: "…we injected different colored tracers in…". This sentence is inaccurate. Instead of "different colored tracers", what the Authors have injected are "different fluorescent tracers" (fast blue, CTB488 and CTB555).

2.3. Results, subheading "Indirect pathway: CDt and cvPut project to cvGPe": Upon delivery of CTB555 in cvGPe and besides a preferential location for labeled neurons in CDt and cvPut, the authors have also found retrogradely-labeled neuron in the centromedian-parafascicular thalamic complex (CM/Pf), the latter likely to be due by partial tracer spread to the putamen. Although this is a feasible explanation, please also note that direct projections arising from CM/Pf reaching the GPe nucleus in rodents have been reported by Kincaid et al. in 1991. See Kincaid AE, Penney JB Jr, Young AB, Newman SW (1991) The globus pallidus receives a projection form the parafascicular nucleus in the rat. Brain Research 553:18-26.

2.4. Results, subheading "A small number of neurons in CDt and cvPut project…": Here the Authors were looking for double-labeled cells, e.g., projection neurons within CDt and cvPut innervating both cdlSNr and cvGPe. To this aim, they compared the percentages of double-labeled neurons in monkey AX (FB injection in cdlSNr and CTB555 injection in cvGPe) with monkey CR (CTB488 injection in cdlSNr and CTB555 injection in cvGPe). Some minor differences in the number of co-labeled cells were found, likely as a result of the different retrograde tracers used. I guess this merits a more in-depth explanation by the Authors.

3. Discussion: Several parts of the Discussion section are too speculative and should be reconsidered by more narrowly relying in anatomical data.

4. Figures: I have some concerns with a number of figures, particularly with the way in which injection sites are illustrated.

4.1. Figure 1, panel B: Up to 3 CTB555 deposits are illustrated in panel B. Please kindly provide a photomicrograph showing the accurate placement of these injections within the CDt.

4.2. Figure 2: To be removed, not required.

4.3. Figure 3: Panels A and C are Nissl-counterstained sections in which the placement of the injection sites (CTB488 in SNr and CTB555 in GPe) are drawn in green and red, respectively. I would rather prefer to see the needle tracks instead of the use of pseudocolor here. Please note that the effective area of tracer uptake should be larger than what is observed in the

Nissl stain. The most accurate choice is to rely on low-power epifluorescent photomicrographs. The same also applies to injection sites illustrated in Figure 9.

4.4. Figure 11: Some press-on-lettering is missed throughout this figure.

5. Final comment: It might also be of interest evaluating whether there is any relationship between the distribution of labeled cells within the CDt and cvPut and the known pattern of patch-matrix compartments of the striatum. If there is any material left, I would like to suggest performing some double-labeling studies with antibodies against the mu-opiod to further disclose patch/matrix compartments.

Reviewer: 2 Charles R. Gerfen (NIMH, USA)

Comments to the Author
Prior work from the Hikosaka lab demonstrates that the tail of the caudate is involved in value coding of visual objects and that the GPe and SNr targets of this area differentially code "bad" and "good" objects. This study confirms the prior study for the tail of the caudate and demonstrates a similar role for the caudal part of the putamen. The anatomical data presented for the most part confirm prior understanding of the organization of the direct and indirect pathway from the striatum by confirming that the organization of these projections from the tail of the caudate and caudal putamen are similar to that of the rostral structures. Much is made of the striatal neurons that are double labeled after injections into both GPe and SNr, suggesting that direct pathway neurons project to both structures. As the authors point out it has been known since 1990 from single neuron axon tracing that direct pathway neurons project to both structures. The retrograde method used in the present study does not provide definitive evidence of whether individual striatal neurons project to both structures as the uptake of the retrogradely transported tracers used may be affected by the amount of axon collateralization. The relative sparseness of double labeled cells could be due either there being a small number of such cells or more likely due to the reliability of the tracer. Although it might not be the intent of the authors to describe that direct pathway neurons project to the GPe as though this is a novel finding, so much emphasis is placed on this finding it comes across that way. Also, it should be pointed out that the while the direct pathway projects to the GPe, this is not to be confused with the segregation of D1 and D2 receptors to the direct and indirect pathways.

The existence of the direct pathway projection to the GPe raises interesting issues regarding the authors' prior findings of the differential "bad" and "good" coding of the GPe and SNr, which is very well discussed and makes this study very worthwhile for publication

Reviewer: 3 Martin Parent (Université Laval Robert-Giffard, Canada)

Comments to the Author
General comments

In this manuscript, Hidetoshi et al. have used anterograde and retrograde tracer injections to label and describe the efferent projections of the caudal striatum using 3 rhesus monkeys. Injections of anterograde tracers placed in the tail of the caudate nucleus (CDt) leads to dense plexuses of axon terminals (doubly labeled for synaptophysin) in the caudal and lateral part of the substantia nigra pars reticulata (cdlSNr) and the caudal and ventral part of the external globus pallidus (cvGPe). These results indicate that CDt neurons are endowed with axons that provide rather restricted projections to both, the cdlSNr and the cvGPe. Authors have previously shown that these basal ganglia area are specialized in visuo-occulomotor behavior. In a second set of experiments, following electrophysiological recordings during behavioral task, authors injected cholera toxin (CTB, used here as antero and retrograde tracer) in the clSNr and cvGPe and found many retrogradely-labeled neurons in the striatum. These neurons projecting to SNr or GPe were intermingled and restricted to tCD and the caudal-ventral region of the putamen (cvPut). A significant proportion of doubly-labeled neurons in the CDt and cvPut were found, suggesting that these neurons project to both, the cdlSNr and cvGPe, through axon collaterals.

Overall, the paper in well written and pleasant to read. The following comments are meant to help the authors in improving their manuscript.

Specific comments

To this reviewer, the Abstract doesn’t properly reflect the interpretation as well as the final conclusion of the manuscript. Indeed, following retrograde tracer injections, authors found a significant proportion (up to 15%) of doubly-labeled neurons in the CDt and cvPut, suggesting that these neurons project to both, the cdlSNr and cvGPe, through a set of axon collaterals. To this reviewer, the proportion of doubly-labeled neurons reported here is significant, considering that a perfect registration of injections into the terminal fields of a single neuron is needed to get this neuron retrogradly-labeled with both tracers. On page 14 / line 17, based on this observation, authors propose a very interesting hypothesis suggesting that EACH CDt neurons may project to both target sites (SNr and GPe), but with biased synaptic effects, susceptible to rapidly change based on learning experience. However in the Abstract, authors mention: “Most neurons were single-labeled, suggesting that they project to either cdlSNr or cvGPe”, “These anatomical results indicate that the signals in CDt and cvPut are sent to

cdlSNr through specialized direct and indirect pathways". These sentences presented in the Abstract should be modified to properly reflect the interpretation of the results.

In the Abstract, authors should also specify the percentage of neurons that were doubly labeled after retrograde tracer injection and specify that this number may be considered as significant considering the importance of false negative results that can be obtained with the use of retrograde labeling approach.

The authors should better outline the novelty of their results presented. For instance, page 9 / line 9, authors mention: "CTB signals were selectively localized as plexuses in cdlSNr and cvGPe, as previously reported (Kim et al., 2017)". To what extent results dealing with anterograde tracer injections that are presented in this manuscript are original?

To this regards, in the Material and methods section, authors only describe retrograde tracer injections in the GPe and SNr. There is no mention of anterograde tracer injections in the CDt.

The last concluding sentence of the Abstract appears to come out of nowhere, since no data regarding saccades or electrophysiological recording are presented in the first part of the Abstract.

Minor comments

Figure 2 should be presented before figure 1. Figure 2 is indeed cited before figure 1 in the manuscript.

Page 9, line 14: "(…) project to the cdlSNr in the direct pathway and to the cvGPe in the indirect pathway." should be replaced by "(…) project to the cdlSNr through the direct pathway and to the cvGPe through the indirect pathway."

Page 9, line 16: "(…) projecting to the direct pathway" should be replaced by "projecting to the cdlSNr". Similarly, "projecting to the indirect pathway" should be replaced by projecting to the cvGPe".

Authors' Response 6 March 2018

Dear Dr. Bolam and Dr. Foxe,

We are very pleased to hear that you consider that this paper will be an important addition to the IBAGS Special Issue. We appreciate editors' and reviewers' comments for this paper, and revised our manuscript and figures according to these comments. To clarify the methods and results and make them easy to understand, we added one table, revised abstract, methods, results and discussion, and reorganized figures. Additionally, we added one figure (Figure 6) for showing the distribution patterns of retrogradely-labeled cells in patch-matrix structures according to reviewer 1's comment. We hope this revision would satisfy editor and reviewers' requests and curiosity.

Best regards,
Hidetoshi Amita

Dear Dr. Amita,

Your manuscript has been reviewed by three external reviewers as well as by the Section Editor, Dr. Yoland Smith, 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 in a revised version of the manuscript before we can consider it further for publication in EJN.

As you can see, the three reviewers found significant merit to your study and we consider that it will be an important addition to the IBAGS Special Issue. However, two of them raised some important technical and interpretative issues that must be dealt with carefully in the revised manuscript. In particular, reviewer 1 raises a series of comments that necessitate more clarity in the methods section and results description. Please, pay careful attention to each of these comments and try to address each of the points in your letter and through changes in the manuscript. Make sure all details about the quantitative approaches used are provided. Also make sure that the methods section is focused on experiments presented in this specific paper. Additional methodological information that is not relevant to studies being presented in this manuscript should be deleted. Some good suggestions are made by the reviewers about the reorganization of figures. Please, pay attention to these as you revise the manuscript.

We also noted the following points that need to be addressed
- Please provide more details for the surgical procedures (anaesthesia, peri-operative care etc.).

Reply: We added more detailed information on anesthesia in Material and methods (page 5): "We implanted a plastic head holder, plastic recording chambers, and scleral search coils under anesthesia induced with ketamine and diazepam maintained using isoflurane gas throughout these surgeries. After 6 weeks of recovery period, we started training and recording. We routinely cleaned the implants by flushing with hydrogen peroxide or a mixture of betadine and saline solution at least three times a week for the health and well-being of subjects."

- Antibody dilutions used for experiment 1 immunofluorescence?

Reply: We added more information on the immunofluorescence procedures for experiment 1 in Materials and methods: Immunofluorescence (page 6): “The sections were preincubated for 30 min in 0.3 % hydrogen peroxide in 0.1 M PBS (pH 7.4) to block endogenous peroxidase, followed by three rinses through 0.1 M PBS, and then 1 hour in blocking solution containing 5% normal goat serum in 0.1 M PBS. The sections were incubated for 18 hours at room temperature in blocking solution containing 2.5% normal goat serum and 0.1% TX-100 with rabbit anti-CTB (1:500) and mouse anti-synaptophysin (1:500) antibody. After three rinses with PBS, the sections were incubated for 2 hours at room temperature with goat anti-rabbit IgG antibody conjugated with alexa fluor 555 (1:200) and goat anti-mouse IgG1 antibody conjugated with alexa fluor 488 (1:200).”

- Please provide a data statement.

Reply: We added data accessibility statement (page 16): “The data and materials presented in the current study can be available upon request by the corresponding author.”

- The reference list needs a careful proof-read.
Reply: We checked the reference list carefully.

When revising the manuscript, please embolden or underline major changes to the text so they are easily identifiable and DO NOT leave 'track change' formatting marks in your paper. Please ensure that you provide a text and a figure file for the Graphical Abstract (as detailed in the instructions below). When carrying out your revisions please refer to the checklist below and visit the EJN author guidelines at www.ejneuroscience.org

When finalized, please upload your complete revised manuscript onto the website, as a Word file (.doc, or .docx). Please also ensure that a complete set of tables and figures is included as separate files, even if these have not changed from the originals. At this stage it is necessary to provide high resolution figures. Please see important instructions below.

Please go into https://mc.manuscriptcentral.com/ejn - Author Centre - manuscripts with decisions where you will find a 'create a revision' link under 'actions'. We ask that you please indicate the way in which you have responded to the points raised by the Editors and Reviewers in a letter. Please upload this response letter as a separate Word (.doc or PDF) file using the file designation "Authors' Response to Reviewers" when uploading your manuscript files. Please DO NOT submit your revised manuscript as a new one. Also, please note that only the Author who submitted the original version of the manuscript should submit a revised version.

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

Comments to the Author
Here the authors evaluated the extent to which basal ganglia direct and indirect pathways are segregated to each other at the level of the most caudally-located basal ganglia territories such as the tail of the caudate nucleus (CDt), the caudal-dorsal-lateral part of the substantia nigra pars reticulata (cdlSNr) and caudo-ventral parts of the external division of the globus pallidus (cvGPe). To this aim, injections of anterograde tracers were achieved in the CDt to evaluate distribution of CDt efferents. Furthermore, retrograde tracers were deposited into cdlSNr and cvGPe to further assess to what extent -if any- CDt parent neurons innervating the cdlSNr and the cvGPe were different cellular populations.

1. Materials and Methods section: I have found this section particularly confusing and very difficult to read. It took me a while to understand simple things like which tracers were injected in experiments 1 and 2 and in which locations. In its present form, the narrative is very hard to understand. If I am right, the authors have used CTB555 for anterograde tract-tracing purposes, this tracer being injected into CDt (experiment 1) and in cvGPe (experiment 2). I guess this section should be rewritten entirely to facilitate an adequate and easy-going understanding.

Reply: Thank you very much for your suggestion which we now feel very important. The main issue was that we used the same or different tracers, the same or different monkeys, and the same or different hemispheres. We thus decided to

present a table (Table 1) which shows these sets of information in two dimensions (monkey's hemisphere vs. injection site). We present the table at the beginning of the second section of Material and methods: Anatomical tracer injection (page 5). This is associated with a simple but essential description: "We injected different tracers in different areas in the CDt-circuit (Table 1) in order to achieve several goals, as shown below…."

1.1. Materials and methods, subheading "Cell-counting": "The images from confocal microscope were inspected systematically, and single labeled (CTB555, CTB488 or FB) or double labeled neurons were identified". Please indicate number of sections analyzed as well as the overall number of counted neurons labeled with each of the tracers used.

Reply: We added the number of sections we analyzed and the number of neurons we detected (page 7):
"In monkey AX, 7 sections (P4, P6, P8, P10, P12, P14 and P16) were analyzed in which 9197 cells (labeled with CTB555) and 4548 cells (with FB) were counted. In monkey CR, 6 sections (P4, P6, P8, P10, P12 and P14) were analyzed in which 825 cells (with CTB488) and 198 cells (with CTB555) were counted."

1.2. In keeping with the former comment and when dealing with neuronal counting, the Authors should explain whether all different tracers did behave the same in terms of number of neurons displaying tracing. In other words, did FB, CTB488 and CTB555 showed a similar efficacy for retrograde neuronal labeling?

Reply: This is a fundamentally important question (related to the above comments), but it is difficult to provide an answer. In monkey AX, the total number of retrogradely labeled cells (we detected) was larger for CTB555 (n=9197) than for FB (n=4548). This suggests at least three possibilities: 1) More neurons in the striatum project to GPe than SNr, 2) The amount of injection was larger in GPe than in SNr, 3) CTB555 was more strongly transported retrogradely than FB. In monkey CR, the total number of retrogradely labeled cells (we detected) was larger for CTB488 (n=825) than for CTB555 (n=198).
Then, one of the multiple possibilities is that the efficacy for retrograde neuronal labeling is different among these tracers (CTB488>CTB555>FB). Nonetheless, this would not be a serious issue to study the ratio of striatal neurons that project to both GPe and SNr.

1.3. Materials and methods, questions regarding "Experiment 1": For experiment 1, authors have used tissue sections available from a former study (as stated in the first paragraph of the Materials and methods section). However, the injected tracer and the place of injection (CTB555 delivered into the CDt) was only mentioned later on in the Results section (subheading "Caudate tail (CDt) directly projects to cdlSNr and cvGPe).

Reply: We agree that it is necessary to explain the method clearly for Experiment 1 as well. We thus added a paragraph (page 5): "In experiment 1, we injected a tracer in CDt in monkey SM to find brain areas that are innervated by CDt (Fig. 1). We used a bi-directional tracer, cholera toxin subunit B conjugated with Alexa Fluor 555 (CTB555; Life technologies). We determined the injection site by recording single neuronal activity. We thus used a custom-made injectrode consisting of an epoxy-coated tungsten microelectrode (200 µm thick; FHC, Bowdoin, ME, USA) for neuronal recording and a silica tube (outer/inner diameter: 155/75 µm; Polymicro Technologies, Phoenix, AZ, USA) for tracer injection. The tracer injection was made after we found several neurons that responded to visual objects selectively and encoded stable values of the objects. We injected 0.3 µl CTB555 (1% in 0.01 M, pH 7.4 phosphate buffer) at a speed of 0.01 µl/min using a 10 µL Hamilton syringe with 30-gauge stainless-steel needle held in a manual infusion pump (Stoelting, Wood Dale, IL, USA). The injection site was 13 and 14 mm posterior to the anterior commissure (P13-14)."

1.4. Although I am not fully sure, it seems that CTB555 was the Authors' main choice for anterograde tract-tracing purposes. Although I am perfectly aware that CTB is a bi-directional tracer, I guess that CTB488 should also produce anterograde labeling of fibers, in a similar fashion than CTB555.

Reply: As shown in Table 1, we injected CTB555 in CDt in monkey SM and cvGPe in monkeys AX and CR. We then used CTB555 for anterograde tracing to study the direct and indirect pathways: CDt to cvGPe and cdlSNr (monkey SM) and cvGPe to cdlSNr (monkey AX). We injected CTB488 in cdlSNr (monkey AX and CR), but this was not used to study anterograde tracing because cdlSNr is the final station of both the direct and indirect pathways.

1.5. Please note that the main aims of this study were (1) to identify cdlSNr and cvGPe territories innervated by CDt efferents and (2) to disclose the precise localization and potential co-labeling of CDt neurons innervating either cdlSNr or cvGPe. In this regard and although I also understand that the authors want to correlate these pathways with the anatomical substrates underlying long-term value coding of visual objects (i.e., stable value memory), it is evident that this manuscript is largely a neuroanatomical study with an inherent value in itself, regardless the functional counterpart. Accordingly, I would like to suggest the Authors removing all parts of the Materials and Methods sections related to Stimuli, Behavioral tasks, and Neuronal recording (i.e., rewrite everything with a narrower focus on tract-tracing experiments).

Reply: We now feel that the physiological-behavioral methods we described are too extensive, compared with the description of their results. We thus decided to remove them entirely, as the reviewer suggested. Yet, we briefly present physiological-behavioral methods and results in some figures (Fig. 2 and Fig. 7) and refer to our previous papers. We hope that reader will be interested in the basic function of the basal ganglia based on the combined information of our current anatomical data and previous physiological data.

2.1. Results, subheading "tracer injections in the stable value-coding regions…". The title of this section is misleading,

because the engagement of cdlSNr and cvGPe regions in stable value memory coding is taken as granted. Please change to “tracer injections in cdlSNr and cvGPe”.

Reply: We changed the subheading, as the reviewer suggested.

2.2. Results, subheading “tracer injections in the stable value-coding…”, second paragraph. Here the authors quoted the following sentence: “…we injected different colored tracers in…”. This sentence is inaccurate. Instead of “different colored tracers”, what the Authors have injected are “different fluorescent tracers” (fast blue, CTB488 and CTB555).

Reply: We modified sentence in Results (page 9): “To address these questions (experiment 2), we first injected different combination of three colored fluorescent tracers (FB, CTB488 and CTB555) in cdlSNr and cvGPe in monkeys AX and CR (Table 1).”

2.3. Results, subheading “Indirect pathway: CDt and cvPut project to cvGPe”: Upon delivery of CTB555 in cvGPe and besides a preferential location for labeled neurons in CDt and cvPut, the authors have also found retrogradely-labeled neuron in the centromedian-parafascicular thalamic complex (CM/Pf), the latter likely to be due by partial tracer spread to the putamen. Although this is a feasible explanation, please also note that direct projections arising from CM/Pf reaching the GPe nucleus in rodents have been reported by Kincaid et al. in 1991. See Kincaid AE, Penney JB Jr, Young AB, Newman SW (1991) The globus pallidus receives a projection form the parafascicular nucleus in the rat. Brain Research 553:18-26.

Reply: Thank you for the important fact. We thus changed the sentence (page 10): “The labeling in CM/Pf might be caused by the tracer spread to the part of Put (Fig. 2B), or by the direct projection of CM/Pf to GPe (Royce & Mourey, 1985; Kincaid et al., 1991; Sadikot et al., 1992; Parent & Parent, 2005).”

2.4. Results, subheading “A small number of neurons in CDt and cvPut project…”: Here the Authors were looking for double-labeled cells, e.g., projection neurons within CDt and cvPut innervating both cdlSNr and cvGPe. To this aim, they compared the percentages of double-labeled neurons in monkey AX (FB injection in cdlSNr and CTB555 injection in cvGPe) with monkey CR (CTB488 injection in cdlSNr and CTB555 injection in cvGPe). Some minor differences in the number of co-labeled cells were found, likely as a result of the different retrograde tracers used. I guess this merits a more in-depth explanation by the Authors.

Reply: This is an important question which is related to the previous comment (1.2.). We thus described the results in different aspects (page 12): “Among neurons retrogradely labeled from cdlSNr, some were retrogradely labeled also from cvGPe: 7.4% (n = 337/4548; monkey AX) and 3.3% (n = 27/825; monkey CR). Among neurons retrogradely labeled from cvGPe, some were retrogradely labeled also from cdlSNr: 3.7% (n = 337/9197; monkey AX) and 13.6% (n = 27/198; monkey CR). Overall, double-labeled neurons among all retrogradely labeled neurons was 2.5% (n = 337/13408) in monkey AX and 2.7% (n = 27/996) in monkey CR.”

3. Discussion: Several parts of the Discussion section are too speculative and should be reconsidered by more narrowly relying in anatomical data.

Reply: Many behavioral-physiological studies focus on the information encoded by neurons in particular brain areas. However, to understand the function of the brain areas, we need to know their input-output connections. Therefore, it is crucial to combine the physiological and anatomical experiments in the same subjects. It is then necessary to discuss the relationship between the physiological and anatomical data. It is also important for future experiments to provide speculative hypotheses. We thus would like to keep the hypotheses based on the combination of the physiological and anatomical experiments. In response to the reviewer’s suggestion, however, we shortened the last paragraph of Discussion.

4. Figures: I have some concerns with a number of figures, particularly with the way in which injection sites are illustrated.

Reply: We rearranged figures and replaced figures for indicating injection sites as the reviewer suggested.

4.1. Figure 1, panel B: Up to 3 CTB555 deposits are illustrated in panel B. Please kindly provide a photomicrograph showing the accurate placement of these injections within the CDt.

Reply: We added a photomicrograph showing the injection site in Fig. 1B.

4.2. Figure 2: To be removed, not required.

Reply: As we describe above, we believe that the integration of physiological and anatomical data is very important. So, we would like to keep the physiological data in Figure 2. We made some changes, however, to de-emphasize the physiological aspect. First, we changed Figure 2 to focus on anatomical data, while physiological data are now used for supporting the anatomical data. Second, we removed one paragraph on physiological data in Results (page 9): “In monkey AX, CTB488 (green) was injected in left cdlSNr (Fig. 3A). Before the injection, we confirmed that neurons around the injection site encoded the stable value: The population activity of 37 neurons in cdlSNr were inhibited by good objects and excited by bad objects (Fig. 3B). CTB555 (red) was injected in right cvGPe (Fig. 3C). Neurons around this injection site also encoded the stable value: the population of 22 neurons in cvGPe were excited by good objects and inhibited by bad objects (Fig. 3D).

These response patterns in cdlSNr and cvGPe were similar as previously reported (Yasuda et al., 2012; Kim et al., 2017), indicating that the tracers were injected in the stable value-coding regions of each target."

4.3. Figure 3: Panels A and C are Nissl-counterstained sections in which the placement of the injection sites (CTB488 in SNr and CTB555 in GPe) are drawn in green and red, respectively. I would rather prefer to see the needle tracks instead of the use of pseudocolor here. Please note that the effective area of tracer uptake should be larger than what is observed in the Nissl stain. The most accurate choice is to rely on low-power epifluorescent photomicrographs. The same also applies to injection sites illustrated in Figure 9.

Reply: We now indicate the needle tracks for tracer injections in some Figures: Fig. 1B (CDt), Fig. 2B (GPe), Fig. 7A (SNr) and Fig. 7B (GPe). However, the needle track for SNr in monkey AX cannot be presented because we injected tracer from a posterior chamber (Fig. 2A).
The pseudo-color areas in Fig. 1B, 2A and 2B indicate the labeled areas by injection in the same Nissl sections. In Fig. 7A and 7B, we combined Nissl sections including needle tracks with epifluorescent photomicrographs of the adjacent sections, because the amounts of tracers were too small to detect the signals in the same Nissl sections.

4.4. Figure 11: Some press-on-lettering is missed throughout this figure.
Reply: Corrected.

5. Final comment: It might also be of interest evaluating whether there is any relationship between the distribution of labeled cells within the CDt and cvPut and the known pattern of patch-matrix compartments of the striatum. If there is any material left, I would like to suggest performing some double-labeling studies with antibodies against the mu-opiod to further disclose patch/matrix compartments.

Reply: Thank you so much for your suggestion. We were also interested in this issue. Encouraged by your suggestion, we performed immunohistochemical staining with antibody against KChIP1 to identify the patch-matrix components. The results had two interesting points. First, patches in CDt and cvPut were very sparse and small. Second, as shown in Fig. 6, retrogradely labeled patches in CDb and Put were labeled by this staining. These results indicate that CDt and cvPut are different from other parts of the striatum in terms of the distribution of patches, and some patches in CDb and putamen project to cdlSNr and cvGPe. We thus added one figure (Fig. 6), Materials and methods (page 7), Results (page 10-11) and Discussion (page 14-15) about 'patch (striosome)'. These results are very interesting and raise many questions which we will address in future experiments.

Reviewer: 2

Comments to the Author
Prior work from the Hikosaka lab demonstrates that the tail of the caudate is involved in value coding of visual objects and that the GPe and SNr targets of this area differentially code "bad" and "good" objects. This study confirms the prior study for the tail of the caudate and demonstrates a similar role for the caudal part of the putamen. The anatomical data presented for the most part confirm prior understanding of the organization of the direct and indirect pathway from the striatum by confirming that the organization of these projections from the tail of the caudate and caudal putamen are similar to that of the rostral structures. Much is made of the striatal neurons that are double labeled after injections into both GPe and SNr, suggesting that direct pathway neurons project to both structures. As the authors point out it has been known since 1990 from single neuron axon tracing that direct pathway neurons project to both structures. The retrograde method used in the present study does not provide definitive evidence of whether individual striatal neurons project to both structures as the uptake of the retrogradely transported tracers used may be affected by the amount of axon collateralization. The relative sparseness of double labeled cells could be due either there being a small number of such cells or more likely due to the reliability of the tracer. Although it might not be the intent of the authors to describe that direct pathway neurons project to the GPe as though this is a novel finding, so much emphasis is placed on this finding it comes across that way. Also, it should be pointed out that the while the direct pathway projects to the GPe, this is not to be confused with the segregation of D1 and D2 receptors to the direct and indirect pathways.
The existence of the direct pathway projection to the GPe raises interesting issues regarding the authors' prior findings of the differential "bad" and "good" coding of the GPe and SNr, which is very well discussed and makes this study very worthwhile for publication.

Reply: Our data showed a small but significant number of neurons projected to both GPe and SNr. These results are relevant to single axon tracing studies which the reviewer commented. We agree that this is an important issue and revised Abstract and Discussion, as shown below.

Abstract (page 2): "These cdlSNr-projecting and cvGPe-projecting neurons were found intermingled in both CDt and cvPut (which we call 'striatum tail'). A small but significant proportion of neurons (< 15%) were double-labeled, indicating that they projected to both cdlSNr and cvGPe."

Discussion (page 14): "However, we also found that a relatively small but significant proportion (< 15%) of the 'striatum tail' neurons project to both cdlSNr and cvGPe. Previous studies using single cell labeling showed that most striatal neurons

project to both GPe and SNr … Thus, each CDt neuron may project to both cdlSNr and cvGPe, but with biased outputs to them (as illustrated in Fig. 11). If the bias is strong, the neuron's soma may be single-labeled with a tracer originating from the dominant output site."

The segregation of D1 and D2 receptors in the direct and indirect pathway-neurons is interesting issue which we will study in future.

Reviewer: 3

Comments to the Author
General comments

In this manuscript, Hidetoshi et al. have used anterograde and retrograde tracer injections to label and describe the efferent projections of the caudal striatum using 3 rhesus monkeys. Injections of anterograde tracers placed in the tail of the caudate nucleus (CDt) leads to dense plexuses of axon terminals (doubly labeled for synaptophysin) in the caudal and lateral part of the substantia nigra pars reticulata (cdlSNr) and the caudal and ventral part of the external globus pallidus (cvGPe). These results indicate that CDt neurons are endowed with axons that provide rather restricted projections to both, the cdlSNr and the cvGPe. Authors have previously shown that these basal ganglia area are specialized in visuo-occulomotor behavior. In a second set of experiments, following electrophysiological recordings during behavioral task, authors injected cholera toxin (CTB, used here as antero and retrograde tracer) in the clSNr and cvGPe and found many retrogradely-labeled neurons in the striatum. These neurons projecting to SNr or GPe were intermingled and restricted to tCD and the caudal-ventral region of the putamen (cvPut). A significant proportion of doubly-labeled neurons in the CDt and cvPut were found, suggesting that these neurons project to both, the cdlSNr and cvGPe, through axon collaterals.

Overall, the paper in well written and pleasant to read. The following comments are meant to help the authors in improving their manuscript.

Specific comments

To this reviewer, the Abstract doesn't properly reflect the interpretation as well as the final conclusion of the manuscript. Indeed, following retrograde tracer injections, authors found a significant proportion (up to 15%) of doubly-labeled neurons in the CDt and cvPut, suggesting that these neurons project to both, the cdlSNr and cvGPe, through a set of axon collaterals. To this reviewer, the proportion of doubly-labeled neurons reported here is significant, considering that a perfect registration of injections into the terminal fields of a single neuron is needed to get this neuron retrogradly-labeled with both tracers. On page 14 / line 17, based on this observation, authors propose a very interesting hypothesis suggesting that EACH CDt neurons may project to both target sites (SNr and GPe), but with biased synaptic effects, susceptible to rapidly change based on learning experience. However in the Abstract, authors mention: "Most neurons were single-labeled, suggesting that they project to either cdlSNr or cvGPe", "These anatomical results indicate that the signals in CDt and cvPut are sent to cdlSNr through specialized direct and indirect pathways". These sentences presented in the Abstract should be modified to properly reflect the interpretation of the results.

Reply: Thank you very much for your suggestion. We thus revised Abstract, emphasizing the significance of double-labeled neurons (page 2): "These cdlSNr-projecting and cvGPe-projecting neurons were found intermingled in both CDt and cvPut (which we call 'striatum tail'). A small but significant proportion of neurons (< 15%) were double-labeled, indicating that they projected to both cdlSNr and cvGPe. These anatomical results suggest that long-term value signals (good vs. bad) are sent from the striatum tail to cdlSNr and cvGPe in a biased (but not exclusive) manner. These connections may play an important role in biasing saccades toward higher-valued objects and away from lower-valued objects."

In the Abstract, authors should also specify the percentage of neurons that were doubly labeled after retrograde tracer injection and specify that this number may be considered as significant considering the importance of false negative results that can be obtained with the use of retrograde labeling approach.

Reply: We now feel that this significant number of double-labeled neurons is important. Thus, we referred to the percentage of double-labeled neurons in Abstract: "A small but significant proportion of neurons (< 15%) were double-labeled, indicating that they projected to both cdlSNr and cvGPe."

The authors should better outline the novelty of their results presented. For instance, page 9 / line 9, authors mention: "CTB signals were selectively localized as plexuses in cdlSNr and cvGPe, as previously reported (Kim et al., 2017)". To what extent results dealing with anterograde tracer injections that are presented in this manuscript are original?

Reply: We agree with the reviewer's comment. We modified sentences in Results to discriminate our current results from our previous results (page 9): "The CTB signals were co-localized with SYN signals (orange signals in Fig. 1D and E, bottom). We confirmed that these CTB-labeled plexuses were indeed not passing fibers but axon terminals from CDt. Hence, CDt had direct projections to cdlSNr and cvGPe."

To this regards, in the Material and methods section, authors only describe retrograde tracer injections in the GPe and SNr. There is no mention of anterograde tracer injections in the CDt.

Reply: The procedure on the anterograde tracer injections in CDt should be mentioned as the reviewer suggested. We added sentences in Materials and methods (page 5): “In experiment 1, we injected a tracer in CDt in monkey SM to find brain areas that are innervated by CDt (Fig. 1; Table. 1). We used a bi-directional tracer, cholera toxin subunit B conjugated with Alexa Fluor 555 (CTB555; Life technologies). We determined the injection site by recording single neuronal activity. We thus used a custom-made injectrode consisting of an epoxy-coated tungsten microelectrode (200 μm thick; FHC, Bowdoin, ME, USA) for neuronal recording and a silica tube (outer/inner diameter: 155/75 μm; Polymicro Technologies, Phoenix, AZ, USA) for tracer injection. The tracer injection was made after we found several neurons that responded to visual objects selectively and encoded stable values of the objects. We injected 0.3 μl CTB555 (1% in 0.01 M, pH 7.4 phosphate buffer) at a speed of 0.01 μl/min using a 10 μL Hamilton syringe attached to a 30-gauge stainless-steel needle held in a manual infusion pump (Stoelting, Wood Dale, IL, USA). The injection site was 13 and 14 mm posterior to the anterior commissure (P13-14).”

The last concluding sentence of the Abstract appears to come out of nowhere, since no data regarding saccades or electrophysiological recording are presented in the first part of the Abstract.

Reply: We agree with your suggestion. We added a sentence mentioning the previous electrophysiological researches in Abstract (page 2): “Our previous studies showed that the caudal basal ganglia control saccades by conveying long-term values (stable values) of many visual objects toward the superior colliculus.”

Minor comments

Figure 2 should be presented before figure 1. Figure 2 is indeed cited before figure 1 in the manuscript.

Reply: We removed the section “Neuronal recording” mentioning Figure 2 in Materials and methods responding to the above suggestion from Reviewer 1. Figure 1 is presented before Figure2 in the manuscript by this revision.

Page 9, line 14: “(…) project to the cdlSNr in the direct pathway and to the cvGPe in the indirect pathway.” should be replaced by “(…) project to the cdlSNr through the direct pathway and to the cvGPe through the indirect pathway.”

Reply: We modified sentence in Results as you suggested (page 9): “The results of experiment 1 suggest that CDt neurons project to cdlSNr through the direct pathway and to cvGPe through the indirect pathway.”

Page 9, line 16: “(…) projecting to the direct pathway” should be replaced by “projecting to the cdlSNr”. Similarly, “projecting to the indirect pathway” should be replaced by projecting to the cvGPe”.

Reply: We modified sentence in Results as you suggested (page 9): “This result raised two questions. First, are the neurons in CDt projecting to cdlSNr and cvGPe spatially segregated?”

2nd Editorial Decision 27 March 2018

Dear Dr. Amita,

Your revised manuscript was re-evaluated by the two original external reviewers as well as by the Section Editor, Dr. Yoland Smith and ourselves. We are pleased to inform you that it will be acceptable for publication in EJN following a few quick minor revisions.

Please attend to the following issues:
1. Your surgical methods are still insufficiently detailed. Please add appropriate details.
2. Dosage of anesthetics should be stated
3. Please provide more details regarding immediate post-operative care
4. Provide concentrations/dilutions of tracers
5. Please provide more details regarding how slices were reconstructed

Thank you for submitting your work to EJN.

Kind regards,

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

Reviews:

EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Reviewer: 1 Jose Lanciego (University of Navarra, Spain)

Comments to the Author
I appreciate very much the efforts undertaken by the authors when replying to my former suggestions, as well as by the changes made in the renewed version of the narrative. The newly-performed stains correlating patch/matrix organization with the location of retrogradely-labeled cells represent a nice added value to the resubmitted manuscript. Furthermore, the location of injection sites is now better illustrated. Although I still have some minor concerns on tracer selection and on comparisons made among different tracers with different tracing capabilities, overall I guess that the manuscript can be accepted in its present form.

Reviewer: 3 Martin Parent (Université Laval Robert-Giffard, Canada)

Comments to the Author
Authors have addressed all concerns raised during the first review and modified their manuscript accordingly.

Authors' Response 28 March 2018

Dear Drs. Foxe and Bolam,

We are pleased to hear that our paper will be acceptable for publication in EJN. We added more detailed information in materials and methods according to your suggestions. We hope this revised manuscript would satisfy your requests.

Best regards,

Hidetoshi Amita

Please attend to the following issues:

1. Your surgical methods are still insufficiently detailed. Please add appropriate details.

Reply: We added detailed information about surgical methods (page 5): "Surgeries for implanting a plastic head holder, plastic recording chambers, and scleral search coils were performed in veterinary operating facility using aseptic procedures. Five minutes after the animal was given atropine (0.04 mg/kg, i.m.), anesthesia was induced with ketamine hydrochloride (10 mg/kg, i.m.) along with diazepam (0.2 mg/kg, i.m.) and maintained using isoflurane gas (1.5 - 3% to effect). Vital signs were monitored throughout the surgeries. The animal's head was placed in a stereotaxic frame. The head holder and chambers were positioned with manipulators onto the skull and affixed with dental cement. The search coil was placed under the conjunctiva."

2. Dosage of anesthetics should be stated

Reply: We stated dosage of anesthetics (page 5): "Five minutes after the animal was given atropine (0.04 mg/kg, i.m.), anesthesia was induced with ketamine hydrochloride (10 mg/kg, i.m.) along with diazepam (0.2 mg/kg, i.m.) and maintained using isoflurane gas (1.5 - 3% to effect)."

3. Please provide more details regarding immediate post-operative care

Reply: We provided more details regarding immediate post-operative care (page 5): "To prevent post-operative swelling after the search coil implantation, antibiotic ointment was placed on the conjunctiva. The post-operative animals were carefully observed. The wound margin was cleaned with betadine-saline solution (2%) and healed by application of antibiotic ointment."

4. Provide concentrations/dilutions of tracers

Reply: We provided concentrations of tracers (page 5): "In monkey CR (Table 1), we injected 0.3 µl CTB488 (1% in 0.01 M, pH 7.4 phosphate buffer) and 0.3 µl CTB555 (1% in 0.01 M, pH 7.4 phosphate buffer) in the same manner except for using a motorized infusion pump (Harvard Apparatus, Holliston, MA, USA)."

5. Please provide more details regarding how slices were reconstructed

Reply: We provided more details regarding how slices were reconstructed (page 6): "The slices were reconstructed according to the locations of the tracer injection sites, the brain structures and the atlas of the rhesus monkey brain."

Reviews:

Reviewer: 1

EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Comments to the Author

I appreciate very much the efforts undertaken by the authors when replying to my former suggestions, as well as by the changes made in the renewed version of the narrative. The newly-performed stains correlating patch/matrix organization with the location of retrogradely-labeled cells represent a nice added value to the resubmitted manuscript. Furthermore, the location of injection sites is now better illustrated. Although I still have some minor concerns on tracer selection and on comparisons made among different tracers with different tracing capabilities, overall I guess that the manuscript can be accepted in its present form.

Reply: Thank you very much for your critical comments and suggestions for our paper. We appreciate your review for improving the quality of this paper. We did not quantitively compare among different tracers with different tracing capabilities in this paper, but this issue will be further studied in future.

Reviewer: 3

Comments to the Author

Authors have addressed all concerns raised during the first review and modified their manuscript accordingly.

Reply: Thank you very much for your useful comments and suggestions for our paper. We appreciate your careful review for improving the quality of this paper.