Manuscript EMBOR-2010-34687

Identification of the AFD neuron as the site of action of the CREB protein in C. elegans thermotaxis

Yukuo Nishida, Takuma Sugi, Mayu Nonomura and Ikue Mori

Corresponding author: Ikue Mori, Nagoya University

Review timeline:

Submission date:	26 December 2010
Editorial Decision:	25 January 2011
Revision received:	22 April 2011
Editorial Decision:	16 May 2011
Additional correspondence (author)	20 May 2011
Additional correspondence (editor)	24 May 2011
Revision received:	24 May 2011
Accepted:	24 May 2011

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 25 January 2011

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, while referees 2 and 3 agree that the study is potentially interesting, referee 1 thinks it is rather preliminary and does provide a limited advance only. Both referees 1 and 2 mention examples of over-interpretations and point out that the conclusion that the cultivation temperature-dependent threshold is vague for crh-1 mutants needs to be supported by experimental data. Importantly, referee 1 also indicates that it should be examined whether CRH-1 is necessary for thermotaxis in AFD neurons, and that it should be determined whether the observed behavioral phenotype of the crh-1 mutant is due to sensory defects in the AFD neuron or due to learning and memory defects. The referee further mentions the lack of important controls and all referees indicate that experimental procedures and reagents are not explained in sufficient detail in the manuscript. We also agree with referee 1 that the title of the manuscript is not appropriate and needs to be changed to more accurately reflect the actual findings.

From the analysis of these comments it is clear that, as it stands, publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given that two referees acknowledge the potential interest and do support publication of your study, I would like to give you the opportunity to address the reviewers concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and

that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Should you decide to embark on such a revision, revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 27,500 characters (including spaces) and, including the maximum of 5 figures, the paper must ultimately fit onto optimally six, and maximally seven, pages of the journal.

When submitting your revised manuscript, please include:

A Microsoft Word file of the manuscript text, editable high resolution TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referee comments. Please also include a two sentence-summary of the manuscript that will appear online on our webpage in case of acceptance of the study for publication.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript Yukuo Nishida et al. show that the C. elegans CREB ortholog, CHR-1, is required only in AFD neurons for thermotaxis. The authors find that CREB mutant worms (crh-1) display abnormal thermotaxis. Rescuing CREB deficiency only in AFD neurons, but not in any of a number of interneurons, restored the impaired behavior. They also show that the thermotactic memory in this paradigm appears to be based on intrinsic neuronal excitability changes rather that synapse strengthening.

The study is rather preliminary at this stage and is also compromised by several experimental limitations listed below. Moreover, given previous reports which have established the role of the C. elegans AFD neurons in thermotaxis and also the involvement of CREB in behavioral plasticity phenomena in several diverse organisms, the scope of the present study is rather narrow and no significant new insight is provided about the molecular basis of CREB function in AFD neurons. In addition the manuscript appears to have been prepared rather hastily and overinterpetations are rife throughout. Finally, the text is laden with numerous syntax and phasing errors, which make it difficult to at times to follow the meaning.

Comments

The quantitative and qualitative assays in fig 2 and 3 show that CRH-1 expression in AFD neurons of chr-1 mutant worms, is sufficient for the worms to display thermotactic behavior, so the worms can learn and remember the temperature in which they can find food when CRH-1 is expressed only in AFD neurons. However, these experiments do not show whether CRH-1 is necessary in AFD neurons of otherwise wt animals, for thermotaxis. In the literature, it has been shown that loss of function mutation of CRH-1 in all cells inhibits thermotaxis (Kimura et al, 2002). The control experiment that is missing here is the knock down of CRH-1 only in AFD neurons (with the rest of the neural circuit expressing CRH-1 normally). This would demonstrate that expression of CRH-1 only in AFD neurons is necessary and sufficient for thermotaxis.

For the experiments shown in figure 4, the authors should use a no - conditioning control. Also their conclusion that "sufficient calcium transient level ensures the neural plasticity of AFD for normal thermotactic behavior..." (page 9, lines 8-12), is unfounded and is rather an overinterpretation of partial data. The defect in calcium transients could well be a mere consequence of CHR-1 deficiency rather that the main cause of behavioral plasticity defects. CHR-1 may influence also other process to bring about behavioral plasticity.

The authors propose a possible mechanism of function of CRH-1 in AFD neuron through regulating the TAX-2 and TAX-4 channels. No experimental evidence whatsoever is offered to support this mechanism. The authors should test whether CHR-1 indeed serves such a regulatory function. In addition, given that CREB activity is dependent on phosphorylation by a variety of kinases, a Western blot analysis using anti-phospho-CREB antibody would be informative.

The authors express crh-1 cDNA in specific neurons of the crh-1(tz2) mutants. For expression in AFD, it is stated that the gcy-8 promoter is used, which drives expression exclusively in AFD neurons. There is no information though about the chr-1 expression in the other neurons reported, i.e. AWC, AIY, AIZ, RIA and ASH. Under which promoter was chr-1 expression driven to ensure exclusive presence of CRH-1 in each of these neurons?

There is no mention of control experiments to verify that chr-1 expression in all the above mentioned neurons has worked properly. Besides the AFD neuron, where a phenotype change in the crh-1(tz2) mutants is obvious, has CRH-1 been detected in those cells, e.g. by fluorescence imaging, or real-time PCR? Also, given that transgenesis in the nematode generally results in overexpression, the authors should confirm that the various phenotypes they observe are not due to overexpression-induced spurious dominant-negative effects.

Page 8, 2nd paragraph: "As a result, the cultivation temperature-dependent threshold at which the animals respond to temperature stimuli appears to be vague in crh-1(tz2) mutants, thus making the impaired threshold, if any, hardly observable". Notwithstanding the unclear phasing of this sentence, it is not at all obvious how the authors reach this conclusion based on the data they have presented in this paragraph.

Is the behavioral phenotype of CHR-1-deficient animals the result of an AFD sensory defect or a learning and memory defect? Given that CREB has been shown to have a general role in learning and memory, the authors should examine whether thermosensation is normal in these animals. In addition, a memory extinguish time-course should be performed.

Other Points

Figure 2 is very cluttered. In panels B-G: Better labeling for the strains that express crh-1 in each kind of neuron. For example, the use of AFDp::crh-1 instead of just AFDp, will make it is easier for the reader to follow the experimental procedure. Also, the results regarding the AWC, AIY, AIZ, RIA and ASH neurons need to be graphed in a separate figure. The way they appear now is very confusing, since two experiments were actually plotted on the same graph. Please, denote the cultivation temperature on the graph. The TTX bars are blurry. The line graphs are thin and faint; they are barely visible. The y-axes have a typographical error (region instead of reasion).

Figure 3: cryophilic and athermotactic movement is not indicated on graph 3A.

Figure 4A: where on the graph do we see the maximum calcium transient levels? What are the two arrows that appear and why are they not explained anywhere in the text? There is no figure legend to inform the reader which line corresponds to which population

Why was the 23-degree cultivation temperature chosen for the calcium recordings when other experiments were done at 20 degrees? Calcium recordings should be performed following a 17- and 20-degree cultivation temperature.

The authors in the discussion jump into conclusions: i) pg 10, paragraph 1, last sentence: can this be inferred on the basis of their findings in figure 4 only? Ii) The concluding sentence of the manuscript is a little bit over the top.

Title: not syntactically correct

Abstract

The first sentence is poorly worded.
Line 4 talks about a "single neuron" that the reader has no idea what it is.
The last sentence is also poorly worded.
There are other minor writing issues in the abstract as well.

Introduction

Why is the complex function of AFD neurons "unexpected"?

CREB is minimally discussed given that it is the target protein of this study.
"By contrast, whether and how the behavioral memory....": there are important findings in the literature regarding the "whether" and the "how" that the authors seem to ignore.

"These observations provide the...., memorize the perceived cultivation temperature": A neuron cannot memorize a cultivation temperature; an organism can.

Last line in page 4 "... whereas restoration-without an s-in other neurons did not": phrasing needs improvement. This is an important point in the paper that is not given enough credit expressed like this.

Last sentence of introduction: improve writing.

Pg 8, line 4 and 5: what is the step-wise temperature warming and the oscillatory manner? These terms were defined neither in the introduction nor here.

Pg 8, paragraph 2, last sentence: poor expression; please rephrase

Results

Last paragraph, pg 7, "AWC, AIY... showed hardly any obvious effect...": rephrase

Pg 5, last paragraph, "... mutants showed the dispersed distribution from colder regions...": what does this mean?

Discussion

Pg 10, paragraph 2, line 3-4: this sentence is difficult to understand

The whole document needs careful proof-reading, to correct numerous syntax and grammar mistakes.

Referee #2 (Revision Comments):

Though I recommend minor revision, there are several points the authors should address as outlined in my report. In particular, the authors should address my point #5 with experiments if this data is not in some supplemental figure that I happen to have missed. In this case, I shall be happy to review a revised version of this manuscript should the authors be invited to do so.

Referee #2 (Remarks to the Author):

Nishida et al present an interesting story characterizing the role of CREB in a worm learning paradigm, i.e. thermotaxis. They show that CREB acts in a single type of sensory neuron AFD rather than in interneurons to regulate behavioral plasticity. This is a surprising yet interesting observation, as people mostly believe CREB should act in interneurons. They also provided functional characterization of AFD in wt and crh-1 mutant worms. Thermotaxis is an interesting behavior, and the Mori group is a pioneer in this field. The experiments are elegantly designed and the data are convincing. And I am therefore happy to support its publication. I have a few minor comments which I hope the authors will be able to address:

1) Abstract: "presents" should be "present"

2) Introduction (Page 3, paragraph 1): The authors state "whether and how the behavioral memory is established at the single cellular level remains enigmatic..." This is an overstatement. Please modify. Numerous studies in other organisms show that cellular plasticity (LTP and LTD) at single neuron level correlates with behavioral plasticity and leaning and memory.

3) Please provide a bar graph summarizing the data in figure 4.

4) Page 8, paragraph 2: The authors state that the ratio changes in wt and crh-1 mutant are "significantly" different. Please provide statistics.

5) Page 8, paragraph 2: The authors state that crh-1 mutant worms have a "vague" cultivation temperature-dependent threshold in AFD calcium imaging assay. Perhaps I missed something from the paper. I could not find the data figure that supports this conclusion. According to this statement, if cultivated at 17C, wt and crh-1 mutant should be similar as shown by behavioral data. But if cultured under 20C and 25C, wt and mutants should be different. Based on this paragraph, it seems to me that the authors have imaged AFD of wt and crh-1 worms grown under different cultivation temperatures. But I cannot find the data (in a supplemental figure I missed?)

6) Unless there are some supplemental files I somehow missed, I cannot find any description of reagents used in the study, e.g. promoters, plasmids, transgenic lines, etc.

Referee #3 (Revision Comments):

This manuscript comes very close to making a simple, important point: that the very interesting CREB transcription factor, implicated in learning and memory in many systems,functions only in the AFD neuron in thermotactic memory. Overall the experiments are great, but I think one additional control is necessary to nail down this fundamental and interesting point. The rescue of the crh-1 thermotactic phenotype is shown to occur only in the AFD when the crh-1 cDNA is expressed in single cell types. A series of appropriate negative controls have been performed, but there might be some low level rescue by expression in some other neurons. Therefore, I suggest mixing the constructs and test rescue to test the (I admit somewhat unlikely) hypothesis that crh-1 has a distributed function in other neurons. This experiment will ensure that you have not missed a broader role, and make the title of the paper appropriate to your nice results.

Before I suggested this experiment, I looked for the injection concentrations in the methods and could not find them. Please state the injection mixes for construction of transgenic animals. If the transgenic strains have been frozen, they should have transgene and strain names so that someone can try to repeat the experiment; if not, state that the strains no longer exist.

A-point-by-point responses to the referees' comments

Referee #1 (Remarks to the Author):

In this manuscript Yukuo Nishida et al. show that the C. elegans CREB ortholog, CHR-1, is required only in AFD neurons for thermotaxis. The authors find that CREB mutant worms (crh-1) display abnormal thermotaxis. Rescuing CREB deficiency only in AFD neurons, but not in any of a number of interneurons, restored the impaired behavior. They also show that the thermotactic memory in this paradigm appears to be based on intrinsic neuronal excitability changes rather that synapse strengthening.

The study is rather preliminary at this stage and is also compromised by several experimental limitations listed below. Moreover, given previous reports which have established the role of the C. elegans AFD neurons in thermotaxis and also the involvement of CREB in behavioral plasticity phenomena in several diverse organisms, the scope of the present study is rather narrow and no significant new insight is provided about the molecular basis of CREB function in AFD neurons. In addition the manuscript appears to have been prepared rather hastily and overinterpetations are rife throughout. Finally, the text is laden with numerous syntax and phasing errors, which make it difficult to at times to follow the meaning.

Comments

The quantitative and qualitative assays in fig 2 and 3 show that CRH-1 expression in AFD neurons of chr-1 mutant worms, is sufficient for the worms to display thermotactic behavior, so the worms can learn and remember the temperature in which they can find food when CRH-1 is expressed only in AFD neurons. However, these experiments do not show whether CRH-1 is necessary in AFD neurons of otherwise wt animals, for thermotaxis. In the literature, it has been shown that loss of function mutation of CRH-1 in all cells inhibits thermotaxis (Kimura et al, 2002). The control experiment that is missing here is the knock down of CRH-1 only in AFD neurons (with the rest of the neural circuit expressing CRH-1 normally). This would demonstrate that expression of CRH-1 only in AFD neurons is necessary and sufficient for thermotaxis.

Response:
We would like to thank the referee for his/her critical reading of our manuscript. We have improved the manuscript as described below.

First of all, we would like to emphasize that there was no description regarding the fact that loss of function mutation of CRH-1 in all cells inhibits thermotaxis in the literature reported by Kimura et al, 2002. We have also convinced that there is no precedent study indicating the role of CRH-1 in thermotaxis.

We constructed the wild-type animals expressing the CRH-1 dominant negative form (CRH-1DN), which carries a point mutation in its phosphorylation site Ser29, to specifically inhibit the endogenous CRH-1 function. When cultivated at 17°C, 20°C and 23°C, the wild-type animals expressing CRH-1DN in AFD or AWC at the injected concentration of 100 ng/ml each appear to exhibit normal thermotactic behavior (Fig 4A-F). We also performed the time-course assay by shifting the cultivation temperature from 17°C to 23°C (Fig 4G). As previously revealed, the time-course assay has been well-known to enable us to estimate the ability to acquire the temperature memory (Gomez et al, 2001; Biron et al, 2006). Importantly, wild-type animals expressing CRH-1DN in AFD at the injected concentration of 100 ng/ml normally migrated toward their cultivation temperature before shifting temperature and at 5 hours after shifting temperature, but they migrated to the colder region and consequently exhibited the lower TTX index value as opposed to wild-type animals until 4 hours after shifting the temperature (Fig 4G). Especially, at 3 and 4 hours after shifting temperature, wild-type animals almost completed acquisition of the ability to migrate toward the new cultivation temperature, whereas animals expressing CRH-1DN in AFD did not (Fig 4G). These results indicated that although wild-type animals expressing CRH-1DN in AFD could ultimately acquire the ability to migrate toward the cultivation temperature at 5 hours after shifting temperature, acquisition of such an ability was significantly slower than that of wild-type animals, as previously observed in other memory-impaired mutants such as *ncs-1* and *dgk-3* mutants (Gomez et al, 2001; Biron et al, 2006). In contrast, wild-type animals expressing CRH-1DN in AWC neuron

at the injected concentration of 100 ng/ml showed the thermotactic behavior similar to wild-type animals after shifting temperature, although wild-type animals expressing CRH-1DN in AFD even at the diluted injected concentration (20 ng/ml) also exhibited the slower acquisition (Fig 4G). These results demonstrated that expression of CRH-1 only in AFD neurons is necessary and sufficient for thermotaxis. We added these new results (Fig 4) in the revised manuscript.

For the experiments shown in figure 4, the authors should use a no - conditioning control. Also their conclusion that "sufficient calcium transient level ensures the neural plasticity of AFD for normal thermotactic behavior..." (page 9, lines 8-12), is unfounded and is rather an overinterpretation of partial data. The defect in calcium transients could well be a mere consequence of CHR-1 deficiency rather that the main cause of behavioral plasticity defects. CHR-1 may influence also other process to bring about behavioral plasticity.

Response:
As mentioned by the referee, we admitted the over-interpretation of the result shown in Figure 4 of the original manuscript. Thus, we softened the conclusion and removed the following sentence, "sufficient calcium transient level ensures the neural plasticity of AFD for normal thermotactic behavior…..". Furthermore, we performed the new experiment according to the referee #2's suggestion, which serves as resolving this referee's concern. When cultivated at 17°C, the AFD neuron of *crh-1(tz2)* mutants exhibited the ratio changes similar to AFD of wild-type animals during both the oscillatory and transient up-and-down temperature changes (Fig 5A, D, G). These results supported the behavioral data, in which *crh-1(tz2)* mutants showed the behavior similar to wild-type animals. When cultivated at 20°C, *crh-1(tz2)* mutants showed the ratio change similar to wild-type animals during the oscillatory warming (Fig 5B), whereas, in up-and-down measurement, AFD of *crh-1(tz2)* mutants exhibited lower ratio changes than that of wild-type animals (Fig 5E). These results suggested that the responsive temperature range of AFD of *crh-1(tz2)* mutants is normal, but magnitude of the calcium concentration changes against the transient up-and-down temperature changes is abnormal. Given that calcium transient level has been well-known to correlate with the neural activity (Kerr et al, 2000; Suzuki et al, 2003; Kuhara et al, 2008), the attenuation of calcium concentration changes reflects the abnormal neural activity, which might cause the behavioral defect of *crh-1(tz2)* mutants. Together with these new results and the original results from the calcium imaging analysis of 23°C-cultivated animals, we suggest that CRH-1 regulates the function of AFD itself through affecting magnitude of its calcium concentration change and that the impairment in this calcium concentration change of AFD, which likely reflects abnormal neuronal activity, might cause the abnormal thermotaxis. We thus provided this description in pages 9-10 and added the new results (Fig 5A, B, D, E, G).

The authors propose a possible mechanism of function of CRH-1 in AFD neuron through regulating the TAX-2 and TAX-4 channels. No experimental evidence whatsoever is offered to support this mechanism. The authors should test whether CHR-1 indeed serves such a regulatory function. In addition, given that CREB activity is dependent on phosphorylation by a variety of kinases, a Western blot analysis using anti-phospho-CREB antibody would be informative.

Response:
The possibility that CRH-1 functions in AFD through regulating the TAX-2 and TAX-4 channels was raised partly based on the result from our calcium imaging analyses as described in page 9 of the revised manuscript and largely on the previous studies (Coburn and Bargmann, 1996; Komatsu et al, 1996; Kimura et al, 2004; Ramot et al, 2008). In addition, we assumed that examination of these mechanisms is next theme for clarifying the mechanism underlying thermotactic behavior. Taking this situation into account, in the original manuscript, we provided in the discussion section the description regarding the speculative mechanisms. On the other hand, we admitted the over-speculation of TAX-2 and TAX-4 mechanisms and phosphorylation mechanism in the discussion section of the original manuscript. Therefore, we removed the description about the possibility of phosphorylation of CRH-1 and softened the description with regard to TAX-2 and TAX-4.

The authors express crh-1 cDNA in specific neurons of the crh-1(tz2) mutants. For expression in AFD, it is stated that the gcy-8 promoter is used, which drives expression exclusively in AFD neurons. There is no information though about the chr-1 expression in the other neurons reported, i.e. AWC, AIY, AIZ, RIA and ASH. Under which promoter was chr-1 expression driven to ensure exclusive presence of CRH-1 in each of these neurons?

Response:
We listed cells expressing GFP fluorescence driven by each *promoter::gfp* fusion gene in supplementary Fig 1A.

There is no mention of control experiments to verify that chr-1 expression in all the above mentioned neurons has worked properly. Besides the AFD neuron, where a phenotype change in the crh-1(tz2) mutants is obvious, has CRH-1 been detected in those cells, e.g. by fluorescence imaging, or real-time PCR? Also, given that transgenesis in the nematode generally results in overexpression, the authors should confirm that the various phenotypes they observe are not due to overexpression-induced spurious dominant-negative effects.

Response:
To confirm whether CRH-1 is detected in AFD, we used serial analysis of gene expression (SAGE) database (http://elegans.bcgsc.bc.ca/), as previously reported. Importantly, consistent with the result from our rescue experiment, SAGE data indicated that CRH-1 is likely expressed in at least AFD. We therefore added this description in the revised manuscript (page 6, line 5 from the bottom). Furthermore, as described above, several lines of new results from CRH-1DN expression experiments reinforces our notion that CRH-1 functions in AFD.

Page 8, 2nd paragraph: "As a result, the cultivation temperature-dependent threshold at which the animals respond to temperature stimuli appears to be vague in crh-1(tz2) mutants, thus making the impaired threshold, if any, hardly observable". Notwithstanding the unclear phasing of this sentence, it is not at all obvious how the authors reach this conclusion based on the data they have presented in this paragraph.

Response:
We admitted the over-interpretation of the results from calcium imaging analysis and should more faithfully describe that result. We therefore replaced the conclusive sentence, “As a result, the cultivation temperature-dependent threshold at which the animals….” in the original manuscript, with the new sentence, “Together with the result from calcium imaging, CRH-1 regulates the function of AFD *per se* through affecting magnitude of its calcium concentration change, although the responsive temperature range does not appear to be defective in *crh-1(tz2)* mutants. The impairment in this calcium concentration change of AFD, which likely reflects abnormal neuronal activity, might cause the abnormal thermotaxis”.

Is the behavioral phenotype of CHR-1-deficient animals the result of an AFD sensory defect or a learning and memory defect? Given that CREB has been shown to have a general role in learning and memory, the authors should examine whether thermosensation is normal in these animals. In addition, a memory extinguish time-course should be performed.

Response:
To address the referee’s critical concern as to whether expression of *crh-1* in AFD indeed contributes to memory or not, we performed the time-course assay using wild-type animals expressing CRH-1 dominant negative form, as suggested by the referee’s comment. Again, as previously revealed by other papers, the time-course assay has been well-known to enable us to estimate the ability to extinguish previous temperature memory and, in other words, the ability to acquire the new temperature memory (Gomez et al, 2001; Biron et al, 2006). We used the wild-type animals expressing CRH-1DN in AFD or AWC at the injected concentration of 100 ng/ml, both of which appear to exhibit normal thermotactic behavior when grown at 17°C, 20°C and 23°C (Fig 4A-F). Wild-type animals expressing CRH-1DN in AFD normally migrated toward their cultivation temperatures before and at 5 hours after shifting temperature, but they migrated to the colder region and consequently exhibited the lower TTX index value as opposed to wild-type animals until 4 hours after shifting the temperature (Fig 4G). Especially, at 3 and 4 hours after shifting temperature, wild-type animals almost completed acquisition of the ability to migrate toward the new cultivation temperature, whereas animals expressing CRH-1DN in AFD did not (Fig 4G). These results indicated that although wild-type animals expressing CRH-1DN in AFD could ultimately acquire the ability to migrate toward the cultivation temperature at 5 hours after shifting temperature, acquisition of such an ability was significantly slower than that of wild-type animals, as previously observed in other memory-impaired mutants such as *ncs-1* and *dgk-3* mutants (Gomez et al, 2001;

Biron et al, 2006). In contrast, wild-type animals expressing CRH-1DN in AWC neuron showed the thermotactic behavior similar to wild-type animals after shifting temperature, although wild-type animals expressing CRH-1DN in AFD even at the diluted injected concentration (20 ng/ml) also exhibited the slower acquisition (Fig 4G). Together with these results, it is suggested that the function of endogenous CRH-1 is important for the rate of the acquisition ability for migration to the new cultivation temperature.

On the other hand, although these results at least supported the notion that expression of *crh-1* in AFD indeed contributes to memory, it is technically difficult to establish the experiment to address whether thermosensation is normal in *crh-1* mutants, partly due to the fact that thermosensation was probably affected by temperature memory itself. To revise the manuscript, we therefore provided this discussion (page 11, lines 2-7) and added new figures for the time-course assay (Fig 4).

Other Points

Figure 2 is very cluttered. In panels B-G: Better labeling for the strains that express crh-1 in each kind of neuron. For example, the use of AFDp::crh-1 instead of just AFDp, will make it is easier for the reader to follow the experimental procedure. Also, the results regarding the AWC, AIY, AIZ, RIA and ASH neurons need to be graphed in a separate figure. The way they appear now is very confusing, since two experiments were actually plotted on the same graph. Please, denote the cultivation temperature on the graph. The TTX bars are blurry. The line graphs are thin and faint; they are barely visible. The y-axes have a typographical error (region instead of reasion).

Response:
According to the referee's suggestion, we provided the better labeling such as *AFDp::crh-1* instead of just AFDp. On the other hand, the separate deposition of the results regarding the AWC, AIY, AIZ, RIA and ASH neurons possibly gives readers arbitrary impression through specifically featuring only the AFD. We therefore separately graphed the results regarding the sensory neurons and interneurons by depositing the AIY, AIZ and RIA neurons as a separate figure. We also denoted the cultivation temperature on the graph, thus clearly illustrated each line and corrected the typographical error.

Figure 3: cryophilic and athermotactic movement is not indicated on graph 3A.

Response:
We indicated cryophilic and athermotactic movement in Fig 3A of the revised manuscript.

Figure 4A: where on the graph do we see the maximum calcium transient levels? What are the two arrows that appear and why are they not explained anywhere in the text? There is no figure legend to inform the reader which line corresponds to which population

Response:
Two arrows that indicate the maximum calcium transient levels were shown not in Figure 4A but in Figure 4B in the original manuscript, and the explanation for these arrows were in the figure legend of Figure 4B in the original manuscript. We decided to remove the arrows and also carefully provided the explanation for which line corresponds to which signal (YFP or CFP?) in figure legend of supplementary Fig 2.

Why was the 23-degree cultivation temperature chosen for the calcium recordings when other experiments were done at 20 degrees? Calcium recordings should be performed following a 17- and 20-degree cultivation temperature.

Response:
Because the most significant behavioral difference between wild-type animals and *crh-1(tz2)* mutants were observed, we chose the 23°C cultivation temperature for the calcium imaging analysis in the original manuscript. As mentioned by this referee and the referee #2, we performed the calcium imaging using 17°C- and 20°C-cultivated animals as additional experiments. As described above, we added these new results in Fig 5A, B, D, E, G in the revised manuscript.

The authors in the discussion jump into conclusions: i) pg 10, paragraph 1, last sentence: can this be inferred on the basis of their findings in figure 4 only? Ii) The concluding sentence of the manuscript is a little bit over the top.

Response:
In revision, we softened the concluding sentence described in paragraph 1 of page 10 in the original manuscript.

Title: not syntactically correct

Response:
We provided the new title, "Identification of the site of action of CREB protein in *C. elegans* thermotaxis."

Abstract
The first sentence is poorly worded.

Response:
We clearly described the first sentence in the revised manuscript.

Line 4 talks about a "single neuron" that the reader has no idea what it is.

Response:
We clearly described the line 4 by using the phrase "AFD thermosensory neuron" in the revised manuscript.

The last sentence is also poorly worded.

Response:
We clearly described the last sentence in the revised manuscript.

There are other minor writing issues in the abstract as well.

Response:
We rewrote the abstract to be much clearly understood.

Introduction
Why is the complex function of AFD neurons "unexpected"?

Response:
We replaced the word "unexpected" with "multiple".

CREB is minimally discussed given that it is the target protein of this study.
"By contrast, whether and how the behavioral memory....": there are important findings in the literature regarding the "whether" and the "how" that the authors seem to ignore.

Response:
We provided more explanations for CREB protein in the revised manuscript, while considering the limitation of manuscript length (27,500 words).

"These observations provide the...., memorize the perceived cultivation temperature": A neuron cannot memorize a cultivation temperature; an organism can.

Response:
We replaced that sentence with the new sentence, "These observations provide the intriguing possibility that the AFD neuron, by itself, possesses the memory about the perceived cultivation temperature".

Last line in page 4 "... whereas restoration-without an s-in other neurons did not": phrasing needs improvement. This is an important point in the paper that is not given enough credit expressed like this.

Response:
We rephrased the sentence.

Last sentence of introduction: improve writing.

Response:
According to the referee's suggestion, we improved the last sentence of introduction.

Pg 8, line 4 and 5: what is the step-wise temperature warming and the oscillatory manner? These terms were defined neither in the introduction nor here.

Response:
We clearly rewrote the step-wise temperature warming and oscillatory manner in the revised manuscript.

Pg 8, paragraph 2, last sentence: poor expression; please rephrase

Response:
We rephrased the last sentence in the revised manuscript.

Results
Last paragraph, pg 7, "AWC, AIY... showed hardly any obvious effect...": rephrase

Response:
We provided more clear description in the revised manuscript.

Pg 5, last paragraph, "... mutants showed the dispersed distribution from colder regions...": what does this mean?

Response:
We rephrased the sentence in the revised manuscript.

Discussion
Pg 10, paragraph 2, line 3-4: this sentence is difficult to understand

Response:
We revised the sentence.

The whole document needs careful proof-reading, to correct numerous syntax and grammar mistakes.

Response:
We did careful proof-reading and carefully checked numerous syntax and grammar mistakes.

Referee #2 (Revision Comments):

Though I recommend minor revision, there are several points the authors should address as outlined in my report. In particular, the authors should address my point #5 with experiments if this data is not in some supplemental figure that I happen to have missed. In this case, I shall be happy to review a revised version of this manuscript should the authors be invited to do so.

Referee #2 (Remarks to the Author):

Nishida et al present an interesting story characterizing the role of CREB in a worm learning paradigm, i.e. thermotaxis. They show that CREB acts in a single type of sensory neuron AFD

rather than in interneurons to regulate behavioral plasticity. This is a surprising yet interesting observation, as people mostly believe CREB should act in interneurons. They also provided functional characterization of AFD in wt and crh-1 mutant worms. Thermotaxis is an interesting behavior, and the Mori group is a pioneer in this field. The experiments are elegantly designed and the data are convincing. And I am therefore happy to support its publication. I have a few minor comments which I hope the authors will be able to address:

1) Abstract: "presents" should be "present"

Response:
We corrected the word.

2) Introduction (Page 3, paragraph 1): The authors state "whether and how the behavioral memory is established at the single cellular level remains enigmatic..." This is an overstatement. Please modify. Numerous studies in other organisms show that cellular plasticity (LTP and LTD) at single neuron level correlates with behavioral plasticity and leaning and memory.

Response:
We admitted the overstatement and therefore softened the sentence. The original sentence was replaced with the sentence, “little is known about the molecular mechanism underlying the establishment of behavioral memory at the single cellular level.”

3) Please provide a bar graph summarizing the data in figure 4.

Response:
We added a bar graph for Figure 5 in the revised manuscript.

4) Page 8, paragraph 2: The authors state that the ratio changes in wt and crh-1 mutant are "significantly" different. Please provide statistics.

Response:
We added statistics.

5) Page 8, paragraph 2: The authors state that crh-1 mutant worms have a "vague" cultivation temperature-dependent threshold in AFD calcium imaging assay. Perhaps I missed something from the paper. I could not find the data figure that supports this conclusion. According to this statement, if cultivated at 17C, wt and crh-1 mutant should be similar as shown by behavioral data. But if cultured under 20C and 25C, wt and mutants should be different. Based on this paragraph, it seems to me that the authors have imaged AFD of wt and crh-1 worms grown under different cultivation temperatures. But I cannot find the data (in a supplemental figure I missed?)

Response:
According to the referee’s suggestion, we measured the calcium transient of wild-type animals and *crh-1(tz2)* mutants cultivated at 17°C or 20°C. Temperature was changed in the moderate oscillatory manner or transient up-and-down manner. The measurement during oscillatory and transient up-and-down temperature changes mainly enables us to know the responsive temperature range and the magnitude of calcium concentration changes of AFD neuron of each animal, respectively. When cultivated at 17°C, the AFD neuron of *crh-1(tz2)* mutants exhibited the ratio changes similar to AFD of wild-type animals during both the oscillatory and transient up-and-down temperature changes (Fig 5A, D, G). These results supported the behavioral data (Fig 2A, G), in which *crh-1(tz2)* mutants showed the behavior similar to wild-type animals. When cultivated at 20°C, *crh-1(tz2)* mutants showed the ratio change similar to wild-type animals during the oscillatory warming (Fig 5B), whereas, in up-and-down measurement, AFD of *crh-1(tz2)* mutants exhibited lower ratio changes than that of wild-type animals (Fig 5E, G). These results suggested that the responsive temperature range of AFD of *crh-1(tz2)* mutants is normal, but magnitude of the calcium concentration changes against the transient up-and-down temperature changes is abnormal. Given that calcium transient level has been well-known to correlate with the neural activity (Kerr et al, 2000; Suzuki et al, 2003; Kuhara et al, 2008), the attenuation of calcium concentration changes reflects the abnormal neural activity, which might cause the behavioral defect of *crh-1(tz2)* mutants. Together with these new results and the original results from the calcium imaging analysis of 23°C-cultivated animals (Fig

5C, F, G), we admitted that the original conclusive sentence, "the cultivation temperature-dependent threshold at which the animals respond to temperature stimuli appears to be vague in *crh-1(tz2)* mutants, …" is overstatement. Faithfully considering the results from calcium imaging analysis, it is reasonable to assume that although the responsive temperature range appears to be not defective in *crh-1(tz2)* mutants, CRH-1 regulates the function of AFD itself through affecting magnitude of its calcium concentration change and that the impairment in this calcium concentration change of AFD, which likely reflects abnormal neuronal activity, might cause the abnormal thermotaxis. In revision, we therefore provided this description in pages 9-10 and added the new results (Fig 5A, B, D, E, G). We would like to thank the referee #2 for the constructive comments and your support for publication of our paper in this journal.

6) Unless there are some supplemental files I somehow missed, I cannot find any description of reagents used in the study, e.g. promoters, plasmids, transgenic lines, etc.

Response:
We regret that the original manuscript did not have supplemental materials. We clearly described the information about promoters, plasmids, transgenic lines, etc and added their lists in supplementary Figure 1.

Referee #3 (Revision Comments):

This manuscript comes very close to making a simple, important point: that the very interesting CREB transcription factor, implicated in learning and memory in many systems,functions only in the AFD neuron in thermotactic memory. Overall the experiments are great, but I think one additional control is necessary to nail down this fundamental and interesting point. The rescue of the crh-1 thermotactic phenotype is shown to occur only in the AFD when the crh-1 cDNA is expressed in single cell types. A series of appropriate negative controls have been performed, but there might be some low level rescue by expression in some other neurons. Therefore, I suggest mixing the constructs and test rescue to test the (I admit somewhat unlikely) hypothesis that crh-1 has a distributed function in other neurons. This experiment will ensure that you have not missed a broader role, and make the title of the paper appropriate to your nice results.

Before I suggested this experiment, I looked for the injection concentrations in the methods and could not find them. Please state the injection mixes for construction of transgenic animals. If the transgenic strains have been frozen, they should have transgene and strain names so that someone can try to repeat the experiment; if not, state that the strains no longer exist.

Response:
According to the referee's suggestion, we constructed the *crh-1(tz2)* transgenic line coordinately expressing *crh-1* cDNA in AWC, AIY, AIZ and RIA, all of which comprise the essential thermotactic neural circuit. We referred this transgenic line as IK874 (supplementary Fig 1B). This strain was cultivated at 17°C, 20°C or 23°C, and we conducted population TTX assay in order to examine if the mixed expression of *crh-1* cDNA rescues the behavioral defect of *crh-1(tz2)* mutants. As shown in Fig 2B, D, F, G-I, expression of *crh-1* in AWC, AIY, AIZ and RIA exerted hardly any observable effect on the thermotactic behavior of *crh-1(tz2)* mutants, suggesting that CRH-1 does not have a distributed function in neurons other than AFD. This result supported our conclusion that CRH-1 functions only in the AFD neuron in thermotactic behavior. In revision, we added this new result in Fig 2B, D, F, G-I. Furthermore, we added the description regarding the injection concentrations in the method section of main text (page 12, lines 13-17). Finally, we provided the list of transgene and strain names in supplementary information (supplementary Fig 1B). We appreciate this referee's fruitful comment and your support for publication of our paper in this journal.

2nd Editorial Decision 16 May 2011

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the enclosed reports from the referees that were asked to assess it.

As you will see, both referees acknowledge that the manuscript has been largely improved. However, referee 1 remains of the opinion that it needs to be excluded that the observed defects after CRH-1 depletion are due to defects in thermosensation. The referee thinks this can be experimentally addressed, and I would like to give you the opportunity to do so in an exceptional second round of revision, given that this is a very important point to support the main conclusion of the study.

EMBO reports has the policy that manuscripts must be accepted within six months after a first decision has been made, which in your case was on the 25th of January. You therefore have about two months left to address this remaining point. If you think this remaining concern cannot be experimentally solved within two months then please let me know and we can discuss alternative solutions.

I look forward to seeing a revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have now addressed most of my concerns from the previous round of review, and the manuscript is more coherent and improved. The main issue that still remains unresolved is whether the observed effects of CRH-1 depletion stem from a defect in thermosensation, rather than in thermotactic memory. I appreciate the argument of the authors that it would be technically challenging to reliably discriminate between the two possibilities, but I would not entirely agree (see for example Genes Dev. 2010, 24: 2365-82 and references therein). This is an important point because if the absence of CRH-1 in AFD neurons disrupts or interferes with their ability of these neurons to sense temperature normally, then the main conclusions of the authors are unfounded. Finally, the manuscript still needs some attention to language and syntax.

Referee #2 (Revision Comments):

The authors have satisfied my comments. I am very happy to support its publication in EMBOR.

Additional correspondence (author) 20 May 2011

First of all, we like to assure the referee by emphasizing again the main conclusion of our manuscript. Our conclusion is described as clearly stated in the manuscript title, "Identification of the site of action of CREB protein in C. elegans thermotaxis", and in the abstract section, "we here found that C. elegans CREB ortholog, CRH-1, is required only in the single bilateral thermosensory neuron AFD for a memory-based behavior". Given that a large number of neuroscientists are enthusiastically seeking the neuron as the site of action of CREB protein using a variety of behavioral paradigm, our conclusion provides the sufficient novelty and advance. Indeed, our results in the manuscript entirely support this conclusion. Given our conclusive sentence in title and abstract section, we have to say that it seems to be too demanding to discriminate the role of CRH-1 between memory and thermosensation.

In Figure 4 of the previous revised manuscript, we clearly demonstrated that CRH-1 regulates the memory in AFD by using the time-course assay and dominant negative form of CRH-1. We have to emphasize again that it is this referee who suggested us to perform this experiment in order to demonstrate the role of CRH-1 in memory. In fact, the time-course assay for thermotactic behavior is a well-established experimental system to investigate the memorizing ability of a mutant (Gomez

et al, 2001; Biron et al, 2006). Thus, our results in the revised manuscript explicitly indicate the importance of CRH-1 in memory of AFD.

In Figure 5A-C of the previous revised manuscript, calcium imaging analysis in each cultivation temperature revealed that AFD in crh-1(tz2) mutants responds in the oscillatory manner by exquisitely recognizing and sensing the subtle temperature differences (~0.045{degree sign}C/sec), and this response is quite similar to AFD in wild-type animals. These results indicate the fact that the ability of AFD to exquisitely sense the temperature is almost normal in crh-1(tz2) mutants.

This referee suggested us to go over the review article by Garrity et al published in Genes & Development 24, 2365-2382 (2010) to design the experiment to determine the role of CREB in either thermosensation or memory. However, I regret to say that the section of C. elegans part in this review article includes misleading statements, such as over-interpretation of the original results in original papers, simply mistaken sentences that do not reflect the original papers, and citation of papers and only a part of the result in a paper which are in favor of authors' papers. (I am quite familiar with this review.) In any case, this referee obviously intends to refer thermoreceptor adaptation experiment (Ramot et al, Nature Neuroscience, 2008). Despite of the referee's suggestion, we think that such experiment would still fail to discern thermosensation and memory as a role of CREB; among several reasons, a main reason is that Ramot et al (2008) could not obtain a solid conclusion from the experiment using electrophysiology.

In summary, we think that the requirement by this referee is unrealistic. Hence, we do not agree to conduct an experiment, which would lead to obtain results that are inconclusive and hardly explicable.

Additional correspondence (editor) 24 May 2011

Thank you for your email explaining why you think additional experiments will not help to distinguish whether the observed thermotactic defect in C. elegans crh-1 mutants is due to defects in thermosensation or memory.

I have read your revised manuscript again and agree that you adequately discuss this issue in the discussion. We can therefore accept your manuscript for publication. However, I would like to suggest some copy-editing changes to the title and abstract, and would like to point out that the legend for figure 2 does not define the error bars. This information needs to be included.

My suggestion for the title is:
Identification of the AFD neuron as the site of action of the CREB protein in C. elegans thermotaxis
I think it is important to mention the AFD neuron, as it is the main result of the paper.

Some minor changes to the abstract:
Behavior is a consequence of computation in neural circuits composed of massive synaptic connections among sensory neurons and interneurons. The CREB protein responsible for learning and memory is expressed in nearly all neurons. Nonetheless, we here found that the C. elegans CREB ortholog, CRH-1, is required only in the single bilateral thermosensory neuron AFD for a memory-based behavior. Restoration of CRH-1 in AFD of CREB-depleted crh-1 mutants completely rescues its thermotactic defect, whereas restorations in other neurons does not. In calcium imaging analyses, the AFD neurons of CREB-depleted crh-1 mutants exhibit an abnormal response to temperature warming. We present a novel platform for analyzing the mechanism of behavioral memory at single cellular resolution within the neural circuit.

Please let me know if you do not agree with any of the suggested changes. You can upload a new version of your manuscript on our website so that we can go ahead and officially accept it.

Yours sincerely,

Editor
EMBO reports

2nd Revision - authors' response 24 May 2011

We are very happy to know that our manuscript (EMBOR-2010-34687V2) **"Identification of the AFD neuron as the site of action of the CREB protein in *C. elegans* thermotaxis"** would be in principle acceptable in EMBO reports. According to your suggestion, we changed the manuscript.

I look forward to hearing from you.

