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Involvement of NMDAR2A tyrosine phosphorylation in depression-related behavior

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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 17 May 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, two of the referees were not able to get back to us with their report as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see the referees are quite positive about the study and would support publication in The EMBO Journal if you could strengthen the manuscript further. One of the major issues raised is that the behavioural characterisation of the YF/YF mice needs to be expanded along the lines suggested. Second, the referees feel that some of the functional experiments that were performed with transfected cells should be performed with neurons from the YF/YF and WT mice. We should thus be happy to consider a revised manuscript if you can address these issues and the other points raised by the referees in an adequate manner.

I should remind you that it is EMBO Journal 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 as well as on the final assessment by the referees.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this paper the authors provide several lines of evidence that Y1325 in the NMDA receptor subunit NR2A is required for the upregulation of function by Src kinase and that Y1325F mice show behavioral manifestations of an anti-depression phenotype. While the paper is generally very well done with elegant experiments there are several points that the authors need to address.

The authors show that Src does not potentiate the function of recombinant NMDA receptor containing NR2A Y1325F. However, differences in kinase effects on recombinantly expressed NMDA receptors and those expressed in neurons have been found previously. Therefore, the authors need to determine whether Src kinases potentiate the function of NMDA receptors in neurons from the YF/YF mice. This should be done to differentiate possible effects on NR2A subunits from those on receptors with NR2B.

Also, the authors allude to "a battery of behavioral tests to examine sensory and motor functions as well as cognition and anxiety of YF/YF mice". However, they only show data for a small subset of tests. To be able to properly appreciate the selectivity of the anti-depression phenotype the authors need to provide information on the other tests that were done. Specifically, because tyrosine kinase effects on NMDA receptors have been implicated in learning and memory in the hippocampus and the amygdala, and in pain, the authors need to determine whether learning/memory and pain behaviors, and the electrophysiological correlates of these, are altered in the YF/YF mice.

Referee #2 (Remarks to the Author):

In this paper, Taniguchi et al reveal a novel phosphorylation site (Y1325) on NMDAR subunit NR2A that is phosphorylated by Src and regulates NMDAR channel activity in a heterologous system. A transgenic mouse (Y1325F) shows no phosphorylation at this site and shows altered behaviour in certain behavioural tasks suggested to be relevant to depression. The work is well done and the results are generally convincing. However, there are a couple of additional experiments that should be carried out prior to publication.

To fully characterise the YF/YF mice and the role of Y1325 phosphorylation in NMDAR function, the authors should be able to demonstrate a Src-dependent increase in NMDAR EPSCs in neurons taken from WT mice that is abolished in neurons from YF/YF mice.

To support the role of Y1325 phosphorylation specifically in depression-related behaviours, the authors should demonstrate a change in phosphorylation at this site in WT mice tested in the tail suspension test, and no change in mice from the open field test.

The authors state in the discussion that they have tested CamKII activation in YF/YF mice. To be able to demonstrate specificity for a pathway downstream of the NMDAR is very important and these data should be shown as part of figure 7.

Minor point:

Page 8, top: change "significantly". No statistical tests were done, hence it is inappropriate to use this word.

Referee #3 (Remarks to the Author):

Tyrosine phosphorylation of NMDAR subunits has been implicated in molecular signaling and also behavioural outputs. To investigate the effects of NR2A phosphorylation Taniguchi et al. identified Tyr-1325 as the major phosphorylation site of this subunit and found that applied Src caused decreased whole cell currents and Ca2+-influx in Y1325F-NR2A expressing HEK cells. Using a knock-in strategy the authors then introduced this mutation to mice that developed normally but showed reduction in NR2a phosphorylation by 40%. Using a combination of biochemical and behavioural experiments this mutant was phenotyped with a focus on abnormalities in the striatum, a brain region involved in depression. Behavioural testing involved Tail suspension, Forced swim, Elevated-Plus and Open-field. YF/YF mutant animals were phenotyped with depression-like behaviour in the absence of other emotional or locomotor abnormalities. The significance of the in vivo findings are underlined by the observation that in the striatum of wt mice Tyr-1325 phosphorylation is induced by the FST but not the EPM. Analyses of changes in downstream signaling reveal the involvement of DARPP-32, ERK and Calcineurin. This is an interesting study providing novel information on how signaling through the NMDAR is influenced by phosphorylation of the NR2A subunit. The experiments are coherent and this mutant mouse is an exciting novel model to study NMDAR function in vivo.

Major points:

1. The biochemical analyses are thorough and reveal a coherent picture of the signaling cascade downstream of NR2A signaling in vivo, yet the behavioural phenotyping requires more attention. While the two standard test of depression-like behaviour produce a robust phenotype, data on locomotion and anxiety need to be strengthened. Overlooked changes in anxiety could confound the interpretation of the phenotype and has merely been assessed by the Open Field and the time spent on open arms in the EPM. There is a great battery of tests to address anxiety-related behaviour more thoroughly. These include the Light-Dark-emergence test, social interactions. There are more readouts for the EPM (Head dips, number of entries in open arms) than just the measure used in the present study.
2. A depression-like phenotype may be part of schizophrenic abnormalities. The authors should therefore address possible molecular changes in PFC neurons and investigate selective attention and sensorimotor gating as prepulse inhibition (PPI) of acoustic startle response.
3. p.10 line 4: the authors claim they had performed sensorimotor and cognitive testing but the paper is lacking the latter aspect. This is important and should be done because cognitive deficits are important features in bipolar disorders.
4. Interesting to see if phosphorylation of all sites in YF/YF containing receptors by Fyn would compensate for the lack of Tyr-1472 sensitivity.
5. The paper lacks any data on neurochemical changes in vivo. Impaired signaling through blocked phosphorylation of NR2a could lead to compensatory increase of extracellular glutamate or other transmitters binding to receptor systems known to cross-talk with the NMDAR system. Since the authors found changes in DARPP-32 which is known to be involved in DA signaling, dopaminergic changes should be investigated, ideally by electrophysiology in brain slices.

Minor points:

1. A cartoon summarizing the signaling pathway through Tyr-1325 phosphorylation would be helpful.
2. p. 11 line 12 The authors refer to Fig. 4 but mean refer to Fig. 5.
3. p.11 line 18 Tyr-1325 is not at all affected rather than 'little'
4. It should be tested if the YF/YF mutants are phosphorylation insensitive in the FST
5. What strain of mice are the ES cells derive from?
6. Please indicate the background of the Cre-deleters.
7. Figs. 6 and 7 could be combined

1st Revision - authors' response 13 August 2009

Point-by-point responses to the reviewers' comments

Referee #1:

1. The authors show that Src does not potentiate the function of recombinant NMDA receptor containing NR2A Y1325F. However, differences in kinase effects on recombinantly expressed NMDA receptors and those expressed in neurons have been found previously. Therefore, the authors need to determine whether Src kinases potentiate the function of NMDA receptors in neurons from the YF/YF mice. This should be done to differentiate possible effects on NR2A subunits from those on receptors with NR2B.

We examined the effect of Src on NMDA receptor channels in medium spiny neurons in acute slices from WT/WT and YF/YF mice (Figure 4 in the revised manuscript). We have found that the amplitude of the NMDA receptor-mediated EPSC was significantly potentiated by application of the Src protein in WT/WT mice but not in YF/YF mice (Figure 4 in the revised manuscript). These data suggest that Tyr-1325 phosphorylation of NR2A is required for the Src-induced potentiation of NMDA receptor currents in medium spiny neurons, which is consistent with the findings from recombinantly expressed NMDA receptors in HEK 293T cells (Figure 2 in the revised manuscript). These are described in page 10, line 1 in the revised manuscript.

2. Also, the authors allude to "a battery of behavioral tests to examine sensory and motor functions as well as cognition and anxiety of YF/YF mice". However, they only show data for a small subset of tests. To be able to properly appreciate the selectivity of the anti-depression phenotype the authors need to provide information on the other tests that were done. Specifically, because tyrosine kinase effects on NMDA receptors have been implicated in learning and memory in the hippocampus and the amygdala, and in pain, the authors need to determine whether learning/memory and pain behaviors, and the electrophysiological correlates of these, are altered in the YF/YF mice.

Following this comment, we have now provided the results of various behavioral tests (the Morris water maze test, contextual fear conditioning test, auditory fear conditioning test, acoustic startle response test, pre-pulse inhibition test, light-dark emergence test, social interaction test, hot plate test, and tail flick test) (Figure 5 and Supplementary Figure 2-4 in the revised manuscript). In these tests, no significant abnormalities were found in YF/YF mice. Thus, we would like to argue that YF/YF mice show a selective impairment in depression-related behavior but not in other general behaviors such as locomotor activity, cognitive function, anxiety-related behavior, and pain behavior. These are described in page 11, line 14 in the revised manuscript. In addition, given that NR2A is not involved in neuropathic pain (Abe et al., Eur J Neurosci, 22: 1445-1454, 2005), it is unlikely that tyrosine phosphorylation of NR2A including Tyr-1325 phosphorylation regulates neuropathic pain.

As to the electrophysiological analysis, it is likely that synaptic plasticity in the hippocampus and the amygdala is normal in YF/YF mice, basing on the finding that hippocampus- and amygdala-dependent learning was normal in YF/YF mice (Supplementary Figure 2). In fact, we did not detect significant abnormalities in the tetanus-induced long-term potentiation in the CA1 region of the hippocampus in YF/YF mice (Supplementary Figure 6 in the revised manuscript). The relevant description is in page 17, line 4.

Referee #2:

1. To fully characterise the YF/YF mice and the role of Y1325 phosphorylation in NMDAR function, the authors should be able to demonstrate a Src-dependent

increase in NMDAR EPSCs in neurons taken from WT mice that is abolished in neurons from YF/YF mice.

This comment is essentially the same as the comment 1 of the referee #1. Please see our response to the comment 1 of the referee #1.

2. To support the role of Y1325 phosphorylation specifically in depression-related behaviours, the authors should demonstrate a change in phosphorylation at this site in WT mice tested in the tail suspension test, and no change in mice from the open field test.

Following this comment, we have added biochemical experiments using brain lysates of mice that were subjected to the tail suspension test and the open field test. We found that the level of Tyr-1325 phosphorylation was increased during the tail suspension test but not during the open field test (Figure 6 and Supplementary Figure 5 in the revised manuscript). The data suggest that Tyr-1325 phosphorylation is selectively involved in depression-related behavior. These are described in page 12, line 22 and page 13, line 4 in the revised manuscript.

3. The authors state in the discussion that they have tested CamKII activation in YF/YF mice. To be able to demonstrate specificity for a pathway downstream of the NMDAR is very important and these data should be shown as part of figure 7.

As suggested by the referee, we have provided biochemical data showing that CaMKII activity is normal in YF/YF mice compared to that in WT/WT mice in Figure 8 of the revised manuscript. These are described in page 15, line 3 in the revised manuscript. Previous studies show that calcineurin is much more sensitive to the increase in Ca2+ concentrations than CaMKII in the postsynaptic cell (Xia and Storm, Nat Rev Neurosci 6: 267-276, 2005; Irvine et al, Trends Neurosci 29: 459-465, 2006). Thus we assume that the lack of the change in CaMKII activity may be explained by the difference in the sensitivity between CaMKII and calcineurin to Ca2+. Src family kinase-mediated Tyr-1325 phosphorylation of the NR2A subunit would induce relatively low and sustained increase in NMDA receptor-mediated currents, which is sufficient to activate calcineurin. In contrast, larger increase in NMDA receptor-mediated currents may be required to affect CaMKII activity. The relevant description is in page 18, line 22.

Minor point:

4. Page 8, top: change "significantly". No statistical tests were done, hence it is inappropriate to use this word.

We have repeated the experiment and found that Y1325F mutation significantly ($p < 0.001$) decreased the tyrosine phosphorylation level of the NR2A subunit (Figure 1C in the revised manuscript). The relevant description is in page 8, line 2.

Referee #3:

Major points:

1. The biochemical analyses are thorough and reveal a coherent picture of the signaling cascade downstream of NR2A signaling in vivo, yet the behavioural phenotyping requires more attention. While the two standard test of depression-like behaviour produce a robust phenotype, data on locomotion and anxiety need to be strengthened. Overlooked changes in anxiety could confound

the interpretation of the phenotype and has merely been assessed by the Open Field and the time spent on open arms in the EPM. There is a great battery of tests to address anxiety-related behaviour more thoroughly. These include the Light-Dark-emergence test, social interactions. There are more readouts for the EPM (Head dips, number of entries in open arms) than just the measure used in the present study.

As suggested by the referee, we have added behavioral analysis, and found that YF/YF mice did not show altered anxiety-like behavior in the light-dark-emergence test, the social interaction test, and the EPM (head dips and number of entries in open arms) as well as in the open field test and the EPM (time spent in open arms) (Figure 5 in the revised manuscript). These results suggest that YF/YF mice showed reduced susceptibility to depression but their anxiety-related behavior is normal. These are described in page 11, line 17 in the revised manuscript.

2. A depression-like phenotype may be part of schizophrenic abnormalities. The authors should therefore address possible molecular changes in PFC neurons and investigate selective attention and sensorimotor gating as prepulse inhibition (PPI) of acoustic startle response.

As suggested by the referee, we have added behavioral experiments and found that PPI of acoustic startle response was unchanged in YF/YF mice compared to that in WT/WT mice (Supplementary Figure 3 in the revised manuscript). Furthermore, given that hypofunction of NMDA receptor-mediated signaling is implicated in the pathophysiology of schizophrenia, we examined the NMDA receptor-mediated signaling in PFC neurons. We found that NMDA receptor-mediated ERK and CREB activations in PFC neurons were unaltered in YF/YF mice compared to those in WT/WT mice (Supplementary Figure 7 in the revised manuscript). Thus, the results suggest that the antidepressant-like behavior of YF/YF mice is not a part of schizophrenic abnormalities. These are described in page 12, line 5 and page 17, line 5 in the revised manuscript.

3. p.10 line 4: the authors claim they had performed sensorimotor and cognitive testing but the paper is lacking the latter aspect. This is important and should be done because cognitive deficits are important features in bipolar disorders.

Following this comment, we have added behavioral analysis and found that the cognitive function of YF/YF mice was normal in the Morris water maze test and the contextual fear conditioning test compared to that of WT/WT mice (Supplementary Figure 2 in the revised manuscript). These are described in page 12, line 5 in the revised manuscript.

4. Interesting to see if phosphorylation of all sites in YF/YF containing receptors by Fyn would compensate for the lack of Tyr-1472 sensitivity.

Tyr-1472 is a major phosphorylation site in the NR2B subunit. Previous studies have shown that the function of NR2A phosphorylation is different from that of Tyr-1472 phosphorylation on NR2B. For example, as we reported previously, NR2B-Y1472F mutant mice show impaired fear-learning in auditory fear conditioning test (Nakazawa et al, EMBO J, 25: 2867-2877, 2006), whereas NR2A null KO mice show normal fear-learning in the same test (Kiyama et al, J Neurosci, 18: 6704-6712, 1998). The role of Fyn-mediated phosphorylation of NR2A, which is likely to regulate the function of NR2A itself, would therefore be different from that of Tyr-1472 phosphorylation on NR2B. Thus, we assume that Fyn-mediated phosphorylation of NR2A cannot compensate for the lack of Tyr-1472 sensitivity.

As to this comment, we are afraid that the referee #3 might have miswritten "Tyr-1325" in NR2A with "Tyr-1472" in NR2B. If so, the following is our response: The current mediated by the Y1325F-NR2A containing receptor was minimally potentiated by the Src protein, nevertheless all tyrosine residues in the NR2A-YF/YF containing receptor should be phosphorylated in the presence of the Src protein in the recording patch pipettes as in vitro phosphorylation study (Figure 1). Thus, the data suggest that phosphorylation of all sites in YF/YF containing receptors cannot compensate for the lack of Tyr-1325 sensitivity.

5. The paper lacks any data on neurochemical changes in vivo. Impaired signaling through blocked phosphorylation of NR2a could lead to compensatory increase of extracellular glutamate or other transmitters binding to receptor systems known to cross-talk with the NMDAR system. Since the authors found changes in DARPP-32 which is known to be involved in DA signaling, dopaminergic changes should be investigated, ideally by electrophysiology in brain slices.

Following this comment, we measured the amounts of monoamines, their metabolites, and amino acids in various brain regions including the hippocampus, hypothalamus, prefrontal cortex, and striatum (Figure 7 in the revised manuscript and data not shown). We found that the levels of dopamine, DOPAC, HVA, 5-HT, 5-HIAA, glutamate and GABA in YF/YF mice were not significantly different from those in WT/WT mice, suggesting that the dopamine, serotonin, and GABA systems are normal in YF/YF mice. These are described in page 13, line 10 in the revised manuscript. To further address this issue, we also measured protein kinase A activity in the striatum, which is regulated by the activity of dopamine receptors and is responsible for DARPP-32 phosphorylation (Supplementary Figure 9 in the revised manuscript). We found that the PKA activity in the striatum of YF/YF mice was not significantly different from that of WT/WT mice. These are described in page 17, line 15 in the revised manuscript. All these results argue that the systems known to cross-talk with the NMDAR system are normal in YF/YF mice. We would like to emphasize that NMDAR-mediated signaling is selectively impaired in YF/YF mice, resulting in antidepressant-like activity (please see the cartoon in Figure 9 in the revised manuscript).

Minor points:

1. A cartoon summarizing the signaling pathway through Tyr-1325 phosphorylation would be helpful.

We have added a cartoon summarizing the signaling pathway through Tyr-1325 phosphorylation (Figure 9 in the revised manuscript).

2. p. 11 line 12 The authors refer to Fig. 4 but mean refer to Fig. 5.

As suggested, "Figure 4" was rewritten as "Figure 6" in the revised manuscript (page 12, line 20).

3. p.11 line 18 Tyr-1325 is not at all affected rather than 'little'.

As suggested, the sentence was amended by replacing "little" with "not at all" (page 13, line 5).

4. It should be tested if the YF/YF mutants are phosphorylation insensitive in the FST.

Following this comment, we have added biochemical experiments using YF/YF mice.

We found that the level of overall tyrosine phosphorylation of Y1325F-NR2A in YF/YF mice is unaltered by the FST (Supplementary Figure 8), suggesting that the tyrosine-phosphorylation sites on NR2A other than Tyr-1325 are insensitive to the FST. These are described in page 17, line 9 in the revised manuscript.

5. What strain of mice are the ES cells derive from?

We used E14.1 (129/Ola-derived) cells, which is described in Materials and Methods of the revised manuscript (page 23, line 18).

6. Please indicate the background of the Cre-deleters.

We used CAG-cre transgenic mice with C57BL/6 background, which is described in Materials and Methods of the revised manuscript (page 23, line 22).

7. Figs. 6 and 7 could be combined.

Figures 6 and 7 in the original manuscript are combined into Figure 8 in the revised manuscript.

2nd Editorial Decision 08 September 2009

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner and that they would thus support publication of the manuscript here.

Still, there is one remaining editorial issue that needs further attention.

Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records).

In the case of the present submission there is one panel that does not fully meet these requirements: figure 1C

I therefore like to kindly ask you to send us a new version of the manuscript that contains a suitably amended version of this figure. I feel that it would also be important to explain the assembly procedure for this panel in the figure legend (i.e. that all lanes come from the same gel). Please be reminded that according to our editorial policies we also need to see the original scans for the figure in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final print version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have satisfied all my concerns about this manuscript.

Referee #2 (Remarks to the Author):

In this revised version of the manuscript, the authors have adequately addressed my previous criticisms. In my opinion, the paper is ready for publication.

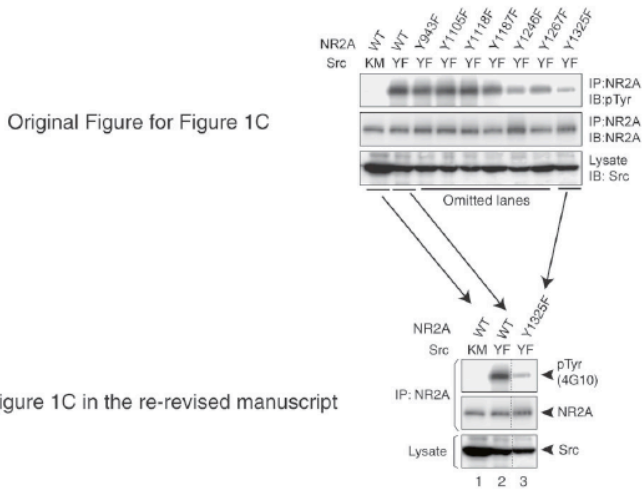
Referee #3 (Remarks to the Author):

The authors have fully revised and provided all information requested by the referee.

2nd Revision - authors' response 09 September 2009

Thank you very much for your letter dated Sep 8, 2009 and for your kind consideration of our manuscript by Taniguchi et al. Following your suggestion, we have amended Figure 1C and the figure legend to Figure 1C (page 37, line 15 in the re-revised text). In addition to the text and figure files, we have also attached the file containing the original figure for Figure 1C to show the assembly procedures.
We believe that the present manuscript is now suitable for publication in The EMBO Journal.
Again, we appreciate very much your kind consideration of our manuscript.

Assembly procedures for Figure 1C



All lanes are from the same gel, but lanes originally present between lanes 2 and 3 have been omitted for brevity.