THE EMBO JOURNAL

Manuscript EMBO-2011-77323

METAPLASTICITY GATED THROUGH DIFFERENTIAL REGULATION OF GLUN2A VERSUS GLUN2B RECEPTORS BY SRC FAMILY KINASES

Kai Yang, Catherine Trepanier, Bikram Sidhu, Yu-Feng Xie, Hongbin Li, Gang Lei, Michael W Salter, Beverley A Orser, Takanobu Nakazawa, Tadashi Yamamoto, Michael F Jackson,

Corresponding author: John MacDonald, University of Toronto

Review timeline:

Submission date:	16 February 2011
Editorial Decision:	21 March 2011
Revision received:	22 September 2011
Editorial Decision:	12 October 2011
Revision received:	08 November 2011
Accepted:	11 November 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 21 March 2011

Thank you for submitting your manuscript to the EMBO Journal. Three referees have now seen your study and their comments are provided below.

The referees appreciate the analysis, but also find that significant revisions are needed in order to strengthen the findings. In particular further direct support for that NR2A-NMDAR current promotes LTP and that NR2B-NMDAR current facilitates LTD is needed. Should you be able to address the concerns raise in full then we would consider a revised manuscript. I should point out that it is our policy to allow a single major round of revision only and it is therefore important to address the concerns raised at this stage. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The NR2 subunit in the NMDA receptors play key roles in the determination of channel kinetics and modulation; in addition, different intracellular signaling pathways appear to be selectively engaged by NMDA receptors containing NR2A or NR2B subunit. Although highly controversial, there is evidence that NR2A and NR2B-containing NMDA receptors have differential contributions to synaptic plasticity (such as LTP and LTD). In this regard, it is important to understand whether NMDARs are subjected to differential and selective regulation by endogenous modulators (such as kinases) and furthermore whether this regulation contributes to synaptic plasticity differentially. Yang and colleagues addressed these two questions using a combination of electrophysiological recording, biochemistry, pharmacology and knockout mice, and in both dissociated neurons and neurons in hippocampal slices. They found that pharmacological activation of PAC1R (via activation of Src kinase) selectively enhanced NR2A-NMDAR current and facilitates LTP while pharmacological activation of D1 dopamine receptors (via activation of Fyn kinase) selectively enhances NR2B-NMDAR current and facilitates LTD. These results are very interesting and could be important if proven correct. However, these results are at odd with some previous published results and the implication for synaptic plasticity is more complicated than the authors have taken.

Major points:

1. The interpretation that enhancement of NR2A NMDARs facilitates LTP while enhancement of NR2B facilities LTD based on the current results are not convincing: these results were based on alterations in synaptic plasticity after incubating hippocampal slices with drugs that activate Src or Fyn kinase. Although NMDARs are affected by these compounds, it is likely that at least a few other targets/pathways that are involved in LTP induction and/or expression are also affected by these compounds. Thus, the observed effects on LTP or LTD could be caused by alterations in these other signaling pathways other than NMDAR itself. The authors should try to show that alteration in NMDARs is the cause of the observed changes in synaptic plasticity. For example, they might be able to use a low dose of NMDAR antagonists to abolish/offset the enhancement in NMDAR current and examine whether this procedure abolishes the observed changes in LTP or LTD.
2. The majority of previous studies showed an enhanced LTP with D1/D5 receptor agonists (including SKF 81927) rather than a reduction in LTP as the authors showed here. In addition, the study by Stamiello and Wagner (Neuropharmacology, 55:871-877, 2008) showed clearly that this facilitation of LTP is abolished by NR2B antagonist. Thus, activation of D1R leads to enhancement in NR2B-NMDARs but the result could be facilitation or inhibition of LTP. The authors need to provide an explanation or resolution to this discrepancy.
3. In the study by Varela et al., (J. Neurosci. 29:3109-3119, 2009), it was shown that SKF 81297 enhanced NR2B-containing NMDARs and depressed NR2A-NMDARs and thus altered the functional ratio of NR2A/NR2B. The authors need to address whether this depression is also seen in their hands and hence whether this affects their interpretation.
4. Why did the ctrl that received 10 Hz give different results in Fig. 4c - 25% depression (upper) but only 10% depression (lower)? The same question applies to 20 Hz. This gives the impression that the degree of LTD / LTP is highly variable between preparations/experiments, and reduces the impact of the conclusions. The authors should explain or try to improve on these data.
5. In Fig. 1c, the set with Ro-25 baseline was not stable prior to addition of PACAP38 (it looks like a continuous increase)
6. In Fig. 2c, should the grey symbols be SKF instead? The increase is very small - 20% and thus it is difficult to say that whether this is functionally meaningful.
7. In Sup Fig. 3, the 1 Hz set is not convincing since the baseline was running down. The 10 Hz set with PACAP appeared to be running up and there was no real potentiation after taking into account of this run-up.
8. Why NR2A KO was not tested in Fig. 4a and b? That would be a nice addition to this experiment.

Minor:
1. In some sample traces, NR2B components appear to be the mainly the sustained components while in others clearly the peak component. Is there any consensus to this?
2. In Fig. 4a and b, a bar rather than arrow should be used to indicate the duration of application of PACAP38 or SKF.

3. In Fig. 4 a and b, scale bars should be shown for the sample traces.
4. In Sup Fig. 4 1 Hz, there does not appear to be any significant difference between the two conditions, albeit claimed so in Fig. 4.
5. Why does NVP increase NMDA current in Fig. 2c,d?
6. Fig. 2 a and b should be discussed prior to discussion of c and d.

Referee #2:

Although remaining highly controversial, differential roles of GluN2A and GluN2B-containing NMDARs in mediating LTP and LTD have recently gained increasing support. However, how the function of these two sub-populations of NMDARs is dynamically regulated by physiological factors such as GPCRs, thereby contributing to metaplasticity remains poorly studied. To this end, the manuscript by Yang et al. fills some of the important gaps by providing interesting results that GPCRs control the direction of synaptic plasticity by differentially regulating the function of synaptic GluN2A or GluN2B through activation of distinct members of the src family. The most striking finding of the manuscript is the selective increase of PAC1R activation leads to Src activation and consequent enhancement of the function of GluN2A containing NMDA receptors, while D1R activation leads to fyn activation and resultant potentiation of GluN2B-containing NMDAR function. Moreover, the increase in synaptic GluN2A contribution is associated with a leftward shift in the frequency-response curve for synaptic plasticity, favoring LTP induction and the increase in GluN2B leads to a change in the rightward direction, favoring LTD. Most of the experiments are well designed and executed; the experimental results are of high quality and convincing. Given its important contributions to the literature in this field, the manuscript can be recommended for publication in the journal provided that the following concerns are fully addressed through an extensive revision.

Major concerns:

1) While the results of differential potentiation of GluN2A and GluN2B by PAC1R-Src and D1-Fyn are very convincing, direct contributions of these differential regulations to metaplastic changes in LTP and LTD inductions remains not established. The authors are encouraged to experimentally demonstrate that the changes in synaptic plasticity can be differentially reversed by the Src-family kinase specific inhibitors (Fyn 39-57or Src40-58) or GluN2 subunit-specific antagonists.

2) From the representative current traces in Fig. 1 and 2, it seems that GluN2A and 2B antagonists appear to respectively affect peak and steady NMDA currents. Given the potentially different contributions to synaptic NMDA components and hence synaptic plasticity, the peak and steady currents should be reevaluated separately.

3) In order to maintain the specificity of the NMDAR subunit preferential antagonists, the authors have carefully chosen to use them at relatively low concentrations (NVP 50 nM, for instance). However, previous studies have suggested that at such low concentrations, these antagonists may not be able to effectively block respective receptors (Wu et al, Mol. Brain, 2007). As the specificity and efficacy of the two inhibitors are critically important for the interpretations of the major results, the authors could perform a set of experiments such as sequential administration of NVP and Ro at these concentrations to demonstrate their specificity and efficacy under the candidate's experimental conditions.

Minor comments:

1. In Fig. 1C, while Ro did not appear to significantly affect PACAP38-induced potentiation of NMDAR currents at 25 min, it did seem to reduce the currents between 10-15 min. Is inhibition at these time points statistically significant? If so, what is the author's interpretation?

2. Fig. 2D, it is interesting that blocking GluN2A receptors potentiated D1-induced enhancement of GluN2B currents. Does this suggest a GluN2A-mediated inhibition of GluN2B modulation? The authors may need to discuss this further.

Referee #3:

Yang, Trepanier, Sidu et al report a form of metaplasticity induced by a GPCR dependent-regulation of specific NMDAR subunits. PAC1R induces the phosphorylation of GluN2A containing receptors via Src kinase activation, and lowered the LTP threshold. D1R induces the phosphorylation of GluN2B via Fyn kinase activation and facilitate LTD induction.
Several previous studies already report the role of PAC1R or D1R in enhancing the NMDA current, the involvement of Src kinase in the NMDA current potentiation by PACAP and D1R, or that GluN2B is the target of Src kinase family activated by D1R (Lei et al 2009 for D1R role on NMDA subunits - not cited by authors), as well the role of D1R in facilitating LTD (for example by Liu et al 2009 (not cited by authors)).
The novelty of this study is to associate the role of Fyn to D1R effect on NMDA receptor, the effects of PAC1R (activated with low concentration of PACAP38) on LTP threshold and the NMDA subunit targeted by PAC1R activation. In general, the results presented in this report are not fully convincing.

Lei, G., N. C. Anastasio, et al. (2009). "Activation of dopamine D1 receptors blocks phencyclidine-induced neurotoxicity by enhancing N-methyl-D-aspartate receptor-mediated synaptic strength." J Neurochem 109(4): 1017-30.
Liu, J., W. Wang, et al. (2009). "Phosphatidylinositol-linked novel D(1) dopamine receptor facilitates long-term depression in rat hippocampal CA1 synapses." Neuropharmacology 57(2): 164-71)

General comments:
1. The paper needs some serious revision and clarification to make it easier to read and make the experiments understandable. Indeed, the experiments are not well described and important information such as the model and species used for each experiment as well as the concentrations of each substance used is missing. Especially, it is written in the material and methods that cell isolation has been performed from rats or mice but it is not known which species has been used in the different experiments.
The duration of drug applications or how the drug is applied (bath solution, patch pipette...) is missing sometimes.
All the n values, especially for the electrophysiology experiments are also missing.
Clearly, the way the experiments have been performed needs to be defined more precisely.
2. The order of the figures does not match with the order they are presented in the manuscript. Especially, figures 2c and d are described before fig 2a and b while it probably makes more sense to present the a and b before the c and d. Furthermore the Supplementary figure 2 should come before the supplemental figure 1 and the figure 2d to introduce Fyn(39-57) and Src(40-58). Supplementary fig. 6 should come after supplementary fig. 5 to be consistent with the order in the text.
3. The figures may be reorganised. Especially, the supplementary fig.3 and 4 and the Figures 4c and 4d could make a figure on its own since they all focus on synaptic plasticity.

Specific comments:
1. Material and method is not complete: the rapid application system is not described, as well as the concentrations of NMDA and coagonist applied (In figure legend 1, the authors write that they doubled the concentration to have 100 µM NMDA and 1 µM glycine, so we should deduce that they used 50 µM NMDA and 0.5 µM Glycine??). This raises the next question to understand why authors used glycine and not D-serine on neurons (Glycine is not specific to NMDAR and can activate other receptors).
2. The concentrations of inhibitors such as NVP-AAM077, ifenprodil or PKI by example are not mentioned either while the selectivity of these compounds clearly depends on the concentration used.
3. It is difficult to understand why most of the experiments performed to identify the signaling pathways involved in NMDAR phosphorylation have been done with an intracellular medium containing 140 mM CsF. Even if fluoride is known to improve quality of the patch recording, it can interfere with many signaling pathway, especially by inhibiting the phosphatases.
4. Also, the authors have used a concentration of zinc of 300 nM to block selectively NR2A component. However, 100 nM has been described to be nearly saturating at blocking NR1/NR2A

(70 % inhibition, that is the maximum inhibition) with a low inhibition of NR1/NR2B. At 300 nM, NR1/NR2A is not more blocked, but NR1/NR2B is blocked at more than 30%. The authors should then reconsider the experiments with a lower concentration.
5. It is also surprising to see that only the peak NMDA current has been studied but not the steady state. In MacDonald et al, BBA 2007, authors wrote "Indeed we suspect that Ipeak is mediated in large part by receptors that only contain NR2A subunits (NR2AA, see below) (but not exclusively) whilst receptors containing only NR2B subunits (NR2BB) contribute more to the steady-state currents". Both parameters should then been analyzed.
6. The shape of the NMDA response (especially presence or not of a peak) is very different at t=0 min between all the NVP experiments (figure 1a, fig 1c, fig 2a and 2c). Could the authors discuss this or choose better example?
7. The authors have also designed a new inhibitor to block Fyn kinase activity specifically, but no proof of the specificity of this inhibitor is given.
8. To ascertain that each receptor effect is due to specific kinase activation, the authors have tested the level of tyrosine phosphorylation for GluN2A and GluN2B without GPCR activation, with GPCR agonist and with GPCR agonist and a kinase inhibitor. But why have the authors only tested the inhibitor they expected to have an effect for each GPCR agonist? In each case we would have expected to see both inhibitors (Fyn and Src). Moreover as PKA has a clear role in D1R induced potentiation of NMDA response, its lack of effect at least on GluN2B tyrosine phosphorylation could have been controlled.
9. All the synaptic plasticity data have to be reanalyzed. Synaptic plasticity has been evaluated through the measure of the amplitude of the EPSP and never the slope. This parameter should not be used as amplitude can be contaminated by population spikes (Such a contamination can be distinguished in the traces illustrated).
10. It is not described in this manuscript how the values of the bar graphs presented in figure 1, 2 and 4 have been calculated. For several experiments, a plateau (and so the maximal value) is not reached within the duration of the experiment (Fig 1a (Src + Ro25-6981); figure 2a (Fyn + Ro25-6981); figure 2a (Fyn + NVP-AAM077); figure 2c...). If the authors have chosen an intermediate time before the maximal amplitude of the effect is reached, they should mention this time and discuss their choice.
11. In the EPSC experiments (figure 4), some data with a GluN2A antagonist (NVP-AAM077) on figure 4a could be of interest to show that the increase on EPSC is mediated via this subunit in this model as well..Applying an NR2A or NR2B antagonist, at least on 1 of the stimulation (10 Hz by example), could also be of interest for the figure 4c-d to show that the effect on metaplasticity is mediated via these independent subunits.
12. Could authors comment the recent article from Lei et al reporting "that activation of dopamine D1 receptors (D1R) with dihydrexidine (DHX) increases serine phosphorylation of the NR1 subunit through protein kinase A activation and tyrosine phosphorylation of the NR2B subunit via Src kinase. DHX also facilitated the synaptic response in cortical slices and this was blocked by an NR2B antagonist."

Many minor points also have to be corrected. For examples:
- the text is not uniform (size of police, indentation, etc., especially in the mat and meth section).
- the different panel in each figure are not uniform (bar graphs, ticks, thickness of lines, police size...).
- the reference to the supplementary data is not consistent (Fig S2A or Supplemenary figure 2A, by example).
- For the paired-pulse facilitation, the supplementary fig.5b is put in reference instead of the supplementary fig.6.
- In the material and methods the authors give the ref 21 (twice and not in good format) while this ref 21 is not the good one.
- The reference list should not contain numbers.
- A space should be added before each ref in the text.
- There is a mistake in the y-axis labelling in fig 4c.
- figure 4a and 4b1: no scale bar for the traces, illustrated
- figure 4d scale bar only for the two upper traces nor the two lower.

1st Revision - authors' response 22 September 2011

Point by Point Responses for Reviewer #1

Referee #1 :

>

> The NR2 subunit in the NMDA receptors play key roles in the determination of channel kinetics and modulation; in addition, different intracellular signaling pathways appear to be selectively engaged by NMDA receptors containing NR2A or NR2B subunit. Although highly controversial, there is evidence that NR2A and NR2B-containing NMDA receptors have differential contributions to synaptic plasticity (such as LTP and LTD). In this regard, it is important to understand whether NMDARs are subjected to differential and selective regulation by endogenous modulators (such as kinases) and furthermore whether this regulation contributes to synaptic plasticity differentially. Yang and colleagues addressed these two questions using a combination of electrophysiological recording, biochemistry, pharmacology and knockout mice, and in both dissociated neurons and neurons in hippocampal slices. They found that pharmacological activation of PAC1R (via activation of Src kinase) selectively enhanced

> NR2A-NMDAR current and facilitates LTP while pharmacological activation of D1 dopamine receptors (via activation of Fyn kinase) selectively enhances NR2B-NMDAR current and facilitates LTD. These results are very interesting and could be important if proven correct. However, these results are at odd with some previous published results and the implication for synaptic plasticity is more complicated than the authors have taken.

>

> Major points:

>

> 1. The interpretation that enhancement of NR2A NMDARs facilitates LTP while enhancement of NR2B facilities LTD based on the current results are not convincing: these results were based on alterations in synaptic plasticity after incubating hippocampal slices with drugs that activate Src or Fyn kinase. Although NMDARs are affected by these compounds, it is likely that at least a few other targets/pathways that are involved in LTP induction and/or expression are also affected by these compounds. Thus, the observed effects on LTP or LTD could be caused by alterations in these other signaling pathways other than NMDAR itself. The authors should try to show that alteration in NMDARs is the cause of the observed changes in synaptic plasticity. For example, they might be able to use a low dose of NMDAR antagonists to abolish/offset the enhancement in NMDAR current and examine whether this procedure abolishes the observed changes in LTP or LTD.

Response to major point number 1

We thank the reviewer for their comments and criticisms. In our opinion this did represent the most important weakness in our study. For this reason we initiated a collaboration with Drs**.** Tadashi Yamamoto and Takanobu Nakazawa using knockin mice, which lack a key site of tyrosine phosphorylation in the GluN2A or the GluN2B subunits, respectively. In these mice the appropriate tyrosines are replaced by phenylalanines (positions 1325 and 1472, GluN2A and GluN2B, respectively). This required us to import breeding pairs from Japan and also required us to breed a sufficient number of these animals for testing. Our primary finding in rats was that, at about 10 Hz stimulation, PACAP applications shifted plasticity towards LTP whilst SKF81297 shifted the relationship in favour of LTD. Therefore, in order to establish that these changes in metaplasticity were indeed due to phosphorylation of NMDARs, and not due to effects on some other target(s), we examined whether or not the appropriate shifts in plasticity could be observed in mice lacking these phosphorylation sites. As anticipated PACAP shifted the responses at 10 Hz towards LTP in wildtype mice but this effect was not present in slices from GluN2AY1325F. Also SKF81297 shifted the responses towards LTD in wildtype mice but this shift was lost in GluN2BY1472F slices. Furthermore, the response to PACAP (enhancement of NMDAR currents) was absence in cells from GluRN2AY1325F slices; and, the response to D1R agonist was lost in cells taken from GluRN2BY1472F knockin mice. These results are now present in a new Fig. 6. These results provide very strong evidence that the phosphorylation of GluN2A and GluN2B subunits is required for the changes in metaplasticity.

> 2. The majority of previous studies showed an enhanced LTP with D1/D5 receptor agonists (including SKF 81927) rather than a reduction in LTP as the authors showed here. In addition, the study by Stamiello and Wagner (Neuropharmacology, 55:871-877, 2008) showed clearly that this

facilitation of LTP is abolished by NR2B antagonist. Thus, activation of D1R leads to enhancement in NR2B-NMDARs but the result could be facilitation or inhibition of LTP. The authors need to provide an explanation or resolution to this discrepancy.

Response to major point number 2.

It is important to reiterate that we do not suggest that enhancing GluNR2B will exclusively enhance LTD nor do we dispute that GluN2B receptors can contribute to LTP. The signals (e.g. calcium influx) mediated by these receptors will potentially trigger effects on either form of synaptic plasticity. Rather, depending upon conditions, the relative contributions of GluN2A versus GluN2B can determine the direction of plasticity. As this is an important point, we have attempted to provide further emphasis for this interpretation in the revised discussion (e.g. first paragraph of revised discussion).

Specific considerations of the studies cited by the referee

In addition, a number of methodological differences between this previous study and ours may account for this discrepancy. These include animal strain (Sprague-Dawley vs Wistar) and age (40-90 vs 21-28 days), slice microdissection (CA3 removed vs intact), as well as numerous other methodological details (e.g. type of stimulating electrode, recording chamber design, perfusion flow rate, fEPSP amplitude, etc).

Differences in slicing methods may also have affected the outcome. In their study, Stramiello and Wagner prepared horizontal brain sections whereas ours were coronal. Assuming that, like us, only transverse sections of the hippocampus were selected (likely, since they claim to have removed the CA3 region from their slices), then their slices would have predominantly been derived from the ventral portion of the hippocampus. This is in contrast to coronal sections where transverse hippocampal slices derive from the dorsal portion of the hippocampus. Evidence suggests that dopaminergic signalling, and indeed more generally hippocampal function, may be segregated along the dorsoventral axis of the hippocampus. Accordingly, such difference may also partially account for differences observed.

The timing of drug application may also have contributed. Stramiello and Wagner applied SKF 30 min prior to HFS and maintained the drug treatment for 30 min after plasticity induction. In contrast, we applied SKF 10 min prior to plasticity induction and terminated the drug treatment immediately afterwards. These notable differences in SKF application timing likely will have resulted in varying degrees of D1R desensitization, known to differentially recruit downstream signalling cascades.

Lastly, since Stramiello did not specifically examine changes in LTD, direct comparison of our respective findings is not possible. Nevertheless, although we did not observe increased LTP, our findings of enhanced LTD by D1R agonist is entirely in-line with previous published reports (Liu et al Neuropharmacology 57; 164-171 (2008). We have added a sentence to the first paragraph of the discussion to acknowledge such differences.

> 3. In the study by Varela et al., (J. Neurosci. 29:3109-3119, 2009), it was shown that SKF 81297 enhanced NR2B-containing NMDARs and depressed NR2A-NMDARs and thus altered the functional ratio of NR2A/NR2B. The authors need to address whether this depression is also seen in their hands and hence whether this affects their interpretation.

Response to major point number 3.

In Varela et al.(2009), the SKF effects on GluN2A- and GluN2B-containing currents were specific to the particular synapses stimulated in the hippocampus: the GluN2A-mediated depression of synaptic responses was observed in entorhinal-CA1 inputs whereas the GluN2B-mediated potentiation of synaptic responses was observed at CA3-CA1 synapses. In our studies, however, we stimulated Schaffer Collateral-CA1 synapses, and as a result we were not able to detect the GluN2A-mediated depression by SKF 81297. This is most likely due to a differential distribution of GluN2A and GluN2B subunits at different hippocampal synapses. According to Varela et al. there is a "predominance of NMDA receptors composed of GluN2A subunits observed in entorhinal–CA1 inputs and a predominance of NMDA receptors composed of GluN2B subunits in CA3–CA1 synapses."

Again there are some major methodological differences between our results and those of Varela et al. Primary among these differences is the age of the rats, Varela used much older animals.

We would point out that using isolated CA1 neurons, in the presence of the GluN2B antagonists, we observed no decrease in the remaining (presumably GluN2A) NMDA evoked current (Figure 2C). Therefore, at this level we were unable to reproduce the report by Varela et al using a synapse free recording system. Furthermore, we examined the potential inhibition of NMDAR-mediated EPSCs by SKF81297 in the slice. Under our recording conditions we did not observe an inhibition of presumed GluN2A-mediated EPSCs, recorded in the presence of Ro 25-6981 (Figure 4B)

> 4. Why did the ctrl that received 10 Hz give different results in Fig. 4c - 25% depression (upper) but only 10% depression (lower)? The same question applies to 20 Hz. This gives the impression that the degree of LTD / LTP is highly variable between preparations/experiments, and reduces the impact of the conclusions. The authors should explain or try to improve on these data.

Response to major point number 4.

Yes, there is variability between slices and that is why we performed experiments in parallel with controls and drug tested preparations. Variability around the theta or transition point from LTD to LTP is to be expected. Recordings from such slices were always interleaved so that bias of selection of slices was minimized. We would also add that at least 2 experimenters, working from different electrophysiology rigs, contributed to the data sets used to construct the BCM plots (Figure 5 in revised manuscript; previously Figure 4). Moreover, the data sets were obtained over a time period of approximately a year and a half. Despite great efforts to standardize methods and electrophysiological setups used within the lab, we have found (anecdotally) that theta can vary slightly from one experimenter to the next (data contributed by K.Y. and B.S.). Any number of factors might contribute to this, most likely minor differences in slices preparation and care. We have also observed some variability at different times of the year and when working from different rodent species (i.e. rat and mouse). Of note, this experience is based not only on the current study but also from past publications (Martin et al., 2010) as well as additional ongoing projects with a number of collaborators. In addition, some of the variability can be attributed to the fact that over the duration of this study the MacDonald lab moved from the University of Toronto, to the Robarts Research Institute at the University of Western Ontario. Here again, some minor differences in theta were observed following the move. Critically, in order to ensure that this variability did not impact on our primary conclusion, we ensured that recordings from control slices were always interleaved with those from drug treated slices (e.g. PACAP or SKF81297). This was consistently undertaken for different experimenters, rigs, rodent species and physical locations. As a result, despite this variability in the absolute value of theta, the characteristic modification of the plasticity modification threshold (i.e. theta) induced by engagement of the signalling cascades described in our study has remained robust and reproducible (i.e. D1R stimulation provokes an increase in theta (more LTD); conversely, PAC_1R stimulation cause its decrease (more LTP). Importantly, we have completed an additional series of recordings in both rat and mouse (transgenic knockin mice). These confirm the salient features previously described (i.e. reciprocal alterations in metaplasticity by select GPCRs). Of note, LTD responses in revised Figure 5C and D are now much more consistent with one another. Accordingly, we feel quite strongly that the impact of our main conclusion is not impacted by this variability.

> 5. In Fig. 1c, the set with Ro-25 baseline was not stable prior to addition of PACAP38 (it looks like a continuous increase)

Response to major point number 5.

The slight apparent "run up" was simply do to the variability between neurons. Unlike many investigators we always average responses from a series of individual neurons treated under the same conditions. As no single neuron is likely to have exactly the same response pattern to activation of a complex signal transduction pathway, there is bound to be some variation in the averaged data. Upon closer statistical examination there was no indication that the responses were continuously increasing. Furthermore our experiences with having made dozens of control recordings without treatment attest to the reliability of the data. We could have chosen just to give a single example but believe the data if more compelling when presented this way, without the exaggeration of showing a response curve for a single neuron. We also examined the effects of applications of Ro in a series of recordings in the absence of applied PACAP38 and can confirm that baseline responses were stable.

> 6. In Fig. 2c, should the grey symbols be SKF instead? The increase is very small - 20% and thus it is difficult to say that whether this is functionally meaningful.

Response to major point number 6.

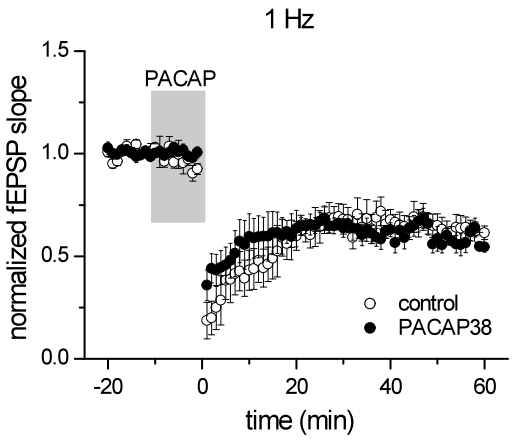
We apologize for the mistake in the figure. The points previously labelled Fyn are in fact the responses to SKF in the absence of either Ro or NVP. The increase reported was significant and every individual neuron responded with an increase following application of SKF (using a non-parametric test this gives a very high value of significance: Mann-Whitey U test, $P<0.001$). Statistically, the increase was also significant as measured by a One-Way ANOVA with Tukey's post-hoc comparison. We are not certain why a 20% increase is considered small as this would undoubtedly contribute towards generating an increased influx of Ca^{2+} through NMDARs, with consequent alteration in downstream signalling. Critically, we would highlight that SKF induced a ~80% increase of isolated NMDAR-EPSCs and produced changes in plasticity, which we now show, were entirely due to changes in GluN2BR function. Accordingly, although the change in isolated pyramidal neurons was proportionally smaller, collectively the data provided supports that it is functionally meaningful.

It is important to note that the distribution of dopaminergic inputs into the hippocampus (from VTA and SNPc) is uneven, being particularly strong in subiculum, hilus and stratum lacunosum-moleculare of the CA1 region. As a result of this uneven distribution, one might expect some CA1 neurons to express more D1 receptors than others - this was apparent in whole-cell recordings from CA1 dissociated neurons where SKF effect was applied: whereas some neurons showed 40 and 50% increases, others showed no effect or very small increases in peak amplitude following SKF application. This is why we have averaged the responses from a total of 9 cells in Fig. 2 (now included the n-value in the figure legend). Overall, the average increase by the end of SKF washout, was about 25%. We believe that such potentiation is functionally significant when one considers the overall impact of dopaminergic modulation of glutamatergic transmission in the hippocampus. For one to consider the functional significance of this effect, one would also need to consider the effect of D2 receptors on NMDAR currents, which we have previously published (Beazely et al., 2006; Kotecha et al., 2002) - D2 receptors inhibit NMDAR currents through a platelet derived growth factor-mediated mechanism. Thus, the overall physiological significance would depend on whether the balance of D1 receptor vs. D2 receptor activation and how this balance would affect overall glutamatergic function in the hippocampus.

We need to reiterate the fact that the responses were taken from individual CA1 neurons dissociated from the hippocampus (and thus do not possess the same synaptic connections as in slice) - the dissociated neurons comprise both synaptic and extrasynaptic receptors (mixed GluN2A/GluN2B responses). When applied in hippocampal slice, SKF produced a much larger increase on the amplitude of NMDAR-mediated EPSCs (mean increase = 1.78). This suggests that the D1R-mediated effect on GluN2B receptors is predominantly synaptic - thus, this effect is diluted in CA1 dissociated neurons (a mixture of synaptic and extrasynaptic NMDA receptors).

> 7. In Sup Fig. 3, the 1 Hz set is not convincing since the baseline was running down. The 10 Hz set with PACAP appeared to be running up and there was no real potentiation after taking into account of this run-up.

We agree with the reviewer; the baseline responses before induction of plasticity should be more stable. To address this concern: **1)** We critically re-examined our existing data set. For the 1 Hz data with PACAP, the rundown could be entirely attributed to one recording. Similarly, for the 10 Hz data with PACAP, run up was observed in 2 recordings. These have now been discarded. **2)** We performed an additional series of recordings (1 Hz: Ctrl n = 1 and PACAP n = 3; 10 Hz: Ctrl n = 4 and PACAP n = 6). The resulting data sets at 1 and 10 Hz have been merged and are now presented in the final figure with the final n values now listed in the figure legend (Figure 5A). In addition, the data points for the BCM plot presented in Figure 5C have been updated to reflect the revised data sets (at both 1 Hz and 10 Hz). Since there is no effect of PACAP at 1 Hz and 100 Hz, we have chosen to omit the full fEPSP vs time (min) plots previously presented in supplemental Figure 3. Nevertheless, the revised data for the 1 Hz set is presented below for the reviewer to evaluate. Note: Additional experiments with SKF 81297 10 Hz: Ctrl n = 5 and SKF n = 4) were also performed in parallel with the supplemental PACAP recordings at 10 Hz, to ensure that reciprocal changes in the direction of plasticity at 10 Hz could still observed in this new series of recordings. This data set has been combined with our previous efforts. The data presented in Figure 5B and C, has been updated accordingly.



Letter Fig. 1.

> 8. Why NR2A KO was not tested in Fig. 4a and b? That would be a nice addition to this experiment.
>

Response to major point number 8.

We agree that these experiments would have been interesting but instead we focused on getting the results from slices from the knockin mice so that we could provide much more direct evidence for the role of the phosphorylation of NMDAR subtypes in the changes in plasticity.
>
> Minor:
> 1. In some sample traces, NR2B components appear to be the mainly the sustained components while in others clearly the peak component. Is there any consensus to this?

Responses of acutely isolated CA1 neurons to rapid applications of NMDA are characterized by a peak response which decays to a quasi-steady state. Our applications of 2 seconds were chosen so that the peak could be readily identified. The peak was determined by visually setting the peak between two cursors. The program (pClamp) then determined the peak and averaged the largest 5 points. We have used this approach in a variety of previous publications (Lu et al., 1999;Huang et al., 2001;Kotecha et al., 2002;Kotecha et al., 2003;Macdonald et al., 2005;Beazely et al., 2008;Xu et al., 2008;Yang et al., 2009).

The apparent steady-state currents were never intended to be measured by us in this study. Firstly, the currents have not reached a true steady-state as the periods of application would have to be much longer to achieve this. Secondly, the quasi-steady state current is determined by the rates of desensitization of NMDARs. Desensitization is dependent upon at least three processes: 1) Conventional agonist-dependent desensitization 2) glycine-dependent desensitization 3) calcium-dependent desensitization (Sather et al., 1990;Sather et al., 1991;Sather et al., 1992;Johnson and Ascher, 1992) (Lu et al., 1999;Lu et al., 2000;Lu et al., 2001). The degree of desensitization of these currents varies substantially between individual cells, thus the relative size of the quasi-steady state currents to the peak is different in each neuron. The shape of the current therefore depends upon the individual cell recorded. In essence the peak currents more closely resemble the synaptic currents, as the quasi steady-state is unlikely to represent a physiological occurrence at the synapse. Therefore, we have measured peak currents and made direct comparisons between these peak currents and the behaviour of synaptic NMDAR currents. We have observed similar regulation of synaptic and peak currents in a variety of signal transduction studies completed over the last ten years (Lu et al., 1999;Huang et al., 2001;Kotecha et al., 2002;Kotecha et al., 2003;Macdonald et al., 2005;Beazely et al., 2008;Xu et al., 2008;Yang et al., 2009).

> 2. In Fig. 4a and b, a bar rather than arrow should be used to indicate the duration of application of PACAP38 or SKF.

This has been changed as requested and, in all new figures, the duration of application is appropriately indicated.

> 3. In Fig. 4 a and b, scale bars should be shown for the sample traces.

The scale bars have now been included in the revised figures.

> 4. In Sup Fig. 4 1 Hz, there does not appear to be any significant difference between the two conditions, albeit claimed so in Fig. 4.

We thank the reviewers for bringing this discrepancy to our attention. We have carefully re-examined the relevant data sets and have now revised Figure 5 (BCM plot: Ctrl and SKF at 1 Hz) to appropriately reflect the 1 Hz data previously included in Supplemental Figure 4. The reviewer is entirely correct, no statistically significant difference is noted between the two data sets (unpaired t-test p = 0.33). We sincerely apologize for this oversight. Supplemental Figure 4 has been removed in the revised manuscript. However, the relevant portions of these figure is provided for comparison below:

Supplemental Figure 4 (from first submission)

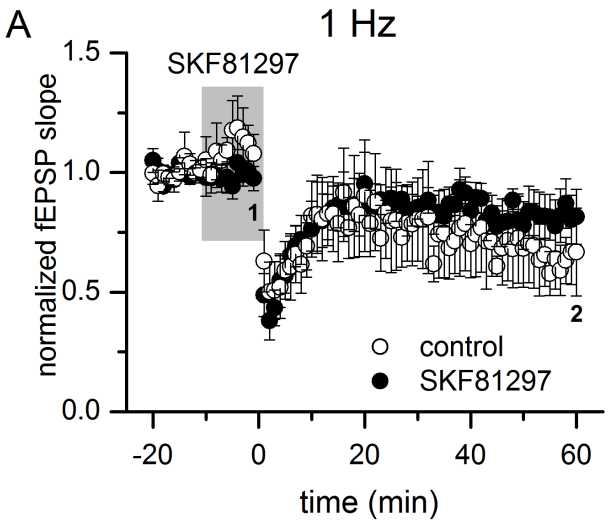
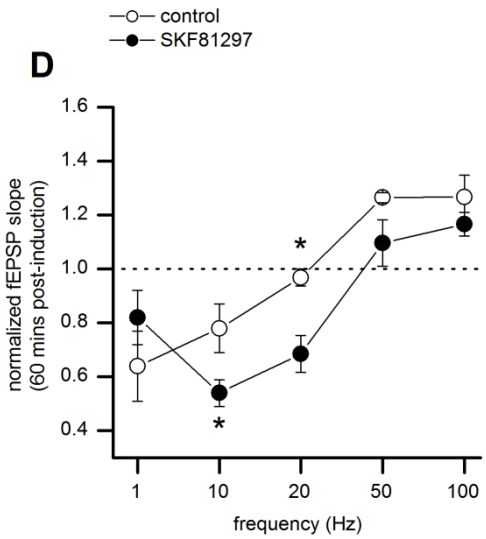


Figure 5 (revised)



> 5. Why does NVP increase NMDA current in Fig. 2c,d?

The NMDAR currents we record are presumably a mixture of responses to both GluN2A and GluN2B receptors. If the currents generated by one subtype are blocked, then the response will largely be mediated by the response to the other subtype. As the responses are normalized to their respective controls it would be anticipated that a GPCR/kinase that enhances the response only to a single subtype of receptor (e.g. blocking GluNR2A) will mean that the proportional enhancement of the remaining current (e.g. GluNR2B) will be larger than controls. Therefore, the effects of the agonist were proportionately larger than for controls. Similar findings were observed with the GluN2AR antagonist Zn^{2+}. More convincingly the enhancements were also proportionately larger in CA1 neurons taken from GluN2A knockout mice. This interpretation is clearly indicated in the results of the revised manuscript: "**Block of GluN2BRs by Ro 25-6981 prevented this enhancement whilst block of GluN2ARs with NVP-AAM077, rather than just preventing, actually increased the proportional enhancement by D1R (Figure 2C and D). The enhancement is anticipated because blocking GluNR2ARs will result in currents generated in greater proportion by GluNR2BRs. Therefore, the effects of the agonist were proportionately larger than for controls. Similar findings were observed with the GluN2AR antagonist Zn^{2+}.**

More convincingly the enhancements were also proportionately larger in CA1 neurons taken from GluN2A knockout mice (Figure 2D).”

In some experiments the concentrations of NMDA and glycine were doubled in order to maintain the amplitude at values similar to the controls (e.g. PACAP38). In these experiments we did not see an additional proportional potentiation (e.g. when Ro25-6981 was used to block GluNR2B, presumably leaving GluNR2A). This was also anticipated as we know from previous work that increasing the concentration of NMDA and glycine will also diminish the relative enhancement of NMDAR mediated currents by Src kinase (see Figure 1 in (Lu et al., 1999)). Furthermore, when concentrations were not increased we did observe a proportional increase in the potentiation (not shown).

> 6. Fig. 2 a and b should be discussed prior to discussion of c and d.
>

The order of discussion has been revised as requested in the new version of the manuscript.

Point by Point Responses for Reviewer #2

> Referee #2:
>
> Although remaining highly controversial, differential roles of GluN2A and GluN2B-containing NMDARs in mediating LTP and LTD have recently gained increasing support. However, how the function of these two sub-populations of NMDARs is dynamically regulated by physiological factors such as GPCRs, thereby contributing to metaplasticity remains poorly studied. To this end, the manuscript by Yang et al. fills some of the important gaps by providing interesting results that GPCRs control the direction of synaptic plasticity by differentially regulating the function of synaptic GluN2A or GluN2B through activation of distinct members of the src family. The most striking finding of the manuscript is the selective increase of PAC1R activation leads to Src activation and consequent enhancement of the function of GluN2A containing NMDA receptors, while D1R activation leads to fyn activation and resultant potentiation of GluN2B-containing NMDAR function. Moreover, the increase in synaptic
> GluN2A contribution is associated with a leftward shift in the frequency-response curve for synaptic plasticity, favoring LTP induction and the increase in GluN2B leads to a change in the rightward direction, favoring LTD. Most of the experiments are well designed and executed; the experimental results are of high quality and convincing. Given its important contributions to the literature in this field, the manuscript can be recommended for publication in the journal provided that the following concerns are fully addressed through an extensive revision.
>
> Major concerns:
>
> 1) While the results of differential potentiation of GluN2A and GluN2B by PAC1R-Src and D1-Fyn are very convincing, direct contributions of these differential regulations to metaplastic changes in LTP and LTD inductions remains not established. The authors are encouraged to experimentally demonstrate that the changes in synaptic plasticity can be differentially reversed by the Src-family kinase specific inhibitors (Fyn 39-57or Src40-58) or GluN2 subunit-specific antagonists.

Response to major concern number 1

We thank the reviewer for their comments and criticisms. In our opinion this did represent the most important weakness in our study. For this reason we initiated a collaboration with Drs**.** Tadashi Yamamoto and Takanobu Nakazawa using knockin mice, which lack a key site of tyrosine phosphorylation in the GluN2A or the GluN2B subunits, respectively. In these mice the appropriate tyrosines are replaced by phenylalanines (positions 1325 and 1472, GluN2A and GluN2B, respectively). This required us to import breeding pairs from Japan and also required us to breed a sufficient number of these animals for testing. Our primary finding in rats was that, at about 10 Hz stimulation, PACAP applications shifted plasticity towards LTP whilst SKF81297 shifted the relationship in favour of LTD. Therefore, in order to establish that these changes in metaplasticity were indeed due to phosphorylation of NMDARs, and not due to effects on some other target(s), we examined whether or not the appropriate shifts in plasticity could be observed in mice lacking these phosphorylation sites. As anticipated PACAP shifted the responses at 10 Hz towards LTP in

wildtype mice but this effect was not present in slices from GluN2AY1325F. Also SKF81297 shifted the responses towards LTD in wildtype mice but this shift was lost in GluN2BY1472F slices. Furthermore, the response to PACAP (enhancement of NMDAR currents) was absence in cells from GluRN2AY1325F slices; and, the response to D1R agonist was lost in cells taken from GluRN2BY1472F knockin mice. These results are now present in a new Fig. 6. These results provide very strong evidence that the phosphorylation of GluN2A and GluN2B subunits is required for the changes in metaplasticity.

> 2) From the representative current traces in Fig. 1 and 2, it seems that GluN2A and 2B antagonists appear to respectively affect peak and steady NMDA currents. Given the potentially different contributions to synaptic NMDA components and hence synaptic plasticity, the peak and steady currents should be reevaluated separately.

>

Response to major concern number 2

Responses of acutely isolated CA1 neurons to rapid applications of NMDA are characterized by a peak response which decays to a quasi-steady state. Our applications of 2 seconds were chosen so that the peak could be readily identified. The peak was determined by visually setting the peak between two cursors. The program (pClamp) then determined the peak and averaged the largest 5 points. We have used this approach in a variety of previous publications (Lu et al., 1999;Huang et al., 2001;Kotecha et al., 2002;Kotecha et al., 2003;Macdonald et al., 2005;Beazely et al., 2008;Xu et al., 2008;Yang et al., 2009).

The apparent steady-state currents were never intended to be measured by us in this study. Firstly, the currents have not reached a true steady-state as the periods of application would have to be much longer to achieve this. Secondly, the quasi-steady state current is determined by the rates of desensitization of NMDARs. Desensitization is dependent upon at least three processes: 1) Conventional agonist-dependent desensitization 2) glycine-dependent desensitization 3) calcium-dependent desensitization (Lu et al., 1999;Lu et al., 2000;Lu et al., 2001) (Sather et al., 1990;Sather et al., 1991;Sather et al., 1992;Johnson et al., 1992). The degree of desensitization of these currents varies substantially between individual cells, thus the relative size of the quasi-steady state currents to the peak is different in each neuron. The shape of the current therefore depends upon the individual cell recorded. In essence the peak currents more closely resemble the synaptic currents, as the quasi steady-state is unlikely to represent a physiological occurrence at the synapse. Therefore, we have measured peak currents and made direct comparisons between these peak currents and the behaviour of synaptic NMDAR currents. We have observed similar regulation of synaptic and peak currents is a variety of signal transduction studies completed over the last ten years (Lu et al., 1999;Huang et al., 2001;Kotecha et al., 2002;Kotecha et al., 2003;Macdonald et al., 2005;Beazely et al., 2008;Xu et al., 2008;Yang et al., 2009)

Lastly, the kinetics of the GluN2A and GluN2B receptor whole cell responses are different but there is always considerable overlap. We speculated in the BBA article that with extremely fast and uniform applications of NMDA the involvement of NR2AA would be emphasized. However, in the vast majority of recordings the difference in kinetics of the receptors simply does not work as a means to separate the NR2AA and NR2BB components and indeed the peak, on average has roughly half and half contributions by these receptors.

The quasi-steady state currents reflects a complex state of desensitization of the various receptors subtypes and this added complexity confounds such measurements.

> 3) In order to maintain the specificity of the NMDAR subunit preferential antagonists, the authors have carefully chosen to use them at relatively low concentrations (NVP 50 nM, for instance). However, previous studies have suggested that at such low concentrations, these antagonists may not be able to effectively block respective receptors (Wu et al, Mol. Brain, 2007). As the specificity and efficacy of the two inhibitors are critically important for the interpretations of the major results, the authors could perform a set of experiments such as sequential administration of NVP and Ro at these concentrations to demonstrate their specificity and efficacy under the candidate's experimental conditions.

>

Response to major concern number 3

Given the concerns about pharmacological specificity we chose to design three different experiments: 1) selectivity of block by low concentrations of NVP, which should partly distinguish between GluNR2A and GluNR2B 2) selectivity by block of a concentration of Zn that will give maximal inhibition of GluNR2A (although not complete) with less effect on GluN2B 3) selectivity

of the signalling pathways in GluN2A-/- mice that are entirely independent of antagonists. For NVP it might be assumed that if NVP were entirely selective for GluNR2A over GluNR2B, then there should be no reduction in the currents recorded from neurons from GluN2A-/- mice. However, we observed a significant reduction by NVP (about 20% not shown). Therefore, we cannot use this drug as suggested by the reviewer. Indeed this concentration of NVP not only blocked GluN2A responses but also partially inhibits those of GluRN2B (or at least presumed to be GluRN2B). We would argue that by using three different experimental approaches we have reached the appropriate interpretation.

> Minor comments:
>
> 1. In Fig. 1C, while Ro did not appear to significantly affect PACAP38-induced potentiation of NMDAR currents at 25 min, it did seem to reduce the currents between 10-15 min. Is inhibition at these time points statistically significant? If so, what is the author's interpretation?
>

Yes, in this case there would appear to be a difference although the ultimate change was not significant as determined by Anova with Bonferroni post-test. It is possible that variations between cells in the relative contributions of GluNR2A and GluNR2B account for this kinetic difference. Unlike many investigators we always average responses from a series of individual neurons treated under the same conditions. As no single neuron is likely to have exactly the same response pattern to activation of a complex signal transduction pathway, there is bound to be some variation in the averaged data. Upon closer statistical examination there was no indication that the responses were continuously increasing, furthermore dozens of control recordings without treatment attest to the reliability of the data. We could have chosen just to give a single example but believe the data if more compelling when presented this way, without the exaggeration of showing a response curve for a single neuron.

> 2. Fig. 2D, it is interesting that blocking GluN2A receptors potentiated D1-induced enhancement of GluN2B currents. Does this suggest a GluN2A-mediated inhibition of GluN2B modulation? The authors may need to discuss this further.

The NMDAR currents we record are presumably a mixture of responses to both GluN2A and GluN2B receptors. If the currents generated by one subtype are blocked, then the response will be entirely mediated by the response to the remaining subtype. As the responses are normalized to their respective controls it would be anticipated that a GPCR/kinase that enhances the response only to a single remaining subtype of receptor would produce a proportionally larger enhancement of the remaining current. Therefore, the effects of the agonist were proportionately larger than for controls. Similar findings were observed with the GluN2AR antagonist Zn^{2+}. More convincingly the enhancements were also proportionately larger in CA1 neurons taken from GluN2A knockout mice. This interpretation is clearly indicated in the results of the revised manuscript: "**Block of GluN2BRs by Ro 25-6981 prevented this enhancement whilst block of GluN2ARs with NVP-AAM077, rather than just preventing, actually increased the proportional enhancement by D1R (Figure 2C and D). The enhancement is anticipated because blocking GluNR2ARs will result in currents generated in greater proportion by GluNR2BRs. Therefore, the effects of the agonist were proportionately larger than for controls. Similar findings were observed with the GluN2AR antagonist Zn^{2+}. More convincingly the enhancements were also proportionately larger in CA1 neurons taken from GluN2A knockout mice (Figure 2D)."**

In other experiments the concentrations of NMDA and glycine were doubled in order to maintain the amplitude at values similar to the controls (e.g. PACAP38). In these experiments we did not see an additional proportional potentiation (e.g. when Ro 25-6981 was used to block GluNR2B, presumably leaving GluNR2A). This was also anticipated as we know from previous work that increasing the concentration of NMDA and glycine will also diminish the relative enhancement of NMDAR mediated currents by Src kinase (see Figure 1 in (Lu et al., 1999)). Furthermore, when concentrations were not increased we did observe a proportional increase in the potentiation (not shown).

We would point out that using isolated CA1 neurons, in the presence of the GluN2BR antagonists, we observed no decrease in the remaining (presumably GluN2AR) NMDA evoked current. Furthermore, we examined the potential inhibition of NMDAR-mediated EPSCs by SKF81297 in the slice. Under our recording conditions we did not observe an inhibition of presumed GluN2AR-mediated EPSCs. On the other hand, cross-talk, whereby GluN2AR

stimulation limits D1R modulation of GluN2B receptors, conceivably could occur (e.g. Ca^{2+} influx through GluN2ARs limits Fyn-mediated phosphorylation of GluN2BRs through activation of Ca^{2+}-dependent tyrosine phosphatase).

Point by Point Responses for Reviewer #3

> Referee #3:

>

> Several previous studies already report the role of PAC1R or D1R in enhancing the NMDA current, the involvement of Src kinase in the NMDA current potentiation by PACAP and D1R, or that GluN2B is the target of Src kinase family activated by D1R (Lei et al 2009 for D1R role on NMDA subunits - not cited by authors), as well the role of D1R in facilitating LTD (for example by Liu et al 2009 (not cited by authors)).

> The novelty of this study is to associate the role of Fyn to D1R effect on NMDA receptor, the effects of PAC1R (activated with low concentration of PACAP38) on LTP threshold and the NMDA subunit targeted by PAC1R activation. In general, the results presented in this report are not fully convincing.

>

> Lei, G., N. C. Anastasio, et al. (2009). "Activation of dopamine D1 receptors blocks phencyclidine-induced neurotoxicity by enhancing N-methyl-D-aspartate receptor-mediated synaptic strength." J Neurochem 109(4): 1017-30.

> Liu, J., W. Wang, et al. (2009). "Phosphatidylinositol-linked novel D(1) dopamine receptor facilitates long-term depression in rat hippocampal CA1 synapses." Neuropharmacology 57(2): 164-71)

General Response to initial comments by review 3

We thank the reviewer for their comments and criticisms. We have extensively revised the manuscript. We have cited the papers indicated and have included them in the discussion. It is important to reiterate that we do not suggest that enhancing GluN2BR will exclusively enhance LTD. The signals (e.g. calcium influx) mediated by these receptors will potentially trigger effects on either form of synaptic plasticity. However, we show that the relative contributions of GluN2AR versus GluN2BR can determine the direction of plasticity. We have attempted to provide further emphasis for this interpretation in the revised discussion. We have also performed additional important experiments on knockin animals (see following).

In order to establish the role of NMDAR phosphorylation in these signaling pathways we initiated a collaboration with Drs**.** Tadashi Yamamoto and Takanobu Nakazawa using knockin mice, which lack a key site of tyrosine phosphorylation in the GluN2A or the GluN2B subunits, respectively. In these mice the appropriate tyrosines are replaced by phenylalanines (positions 1325 and 1472, GluN2A and GluN2B, respectively). This required us to import the breeding mice from Japan and also required us to breed a sufficient number of these animals for testing. Our primary finding in rats was that at about 10 Hz stimulation PACAP applications shifted plasticity towards LTP whilst SKF81297 shifted the relationship in favour of LTD. Therefore, in order to establish that these changes in metaplasticity were indeed due to phosphorylation of NMDARs, and not due to effects some other target(s) we examined whether or not the appropriate shifts in plasticity could be observed in mice lacking these phosphorylation sites. As anticipated PACAP shifted the responses at 10 Hz towards LTP in wildtype mice but this effect was not present in slices from GluN2AY1325F. Also SKF81297 shifted the responses towards LTD in wildtype mice but this shift was lost in GluN2BY1472F slices. Furthermore, the response to PACAP (enhancement of NMDAR currents) was absence in cells from GluN2AY1325F slices; and, the response to D1R agonist was lost in cells taken from GluN2BY1472F knockin mice. These results are now present in a new Fig. 6. These results provide very strong evidence that the phosphorylation of GluN2A and GluN2B subunits is required for the changes in metaplasticity.

More generally we feel it is important to address criticism aimed at the novelty of our findings. We agree with the reviewer that past studies already report ostensibly similar findings demonstrating the regulation of NMDARs and plasticity by GCPR-initiated cell signalling. We apologize if our previous draft gave the impression that novelty lay strictly in the general elucidation of these cascades. Rather, the true novelty of our study resides in the demonstration that NMDAR subpopulations (i.e. GluN2A- vs GluN2B-subunit containing), present within the hippocampus, are

targeted selectively by distinct signalling cascades thus allowing them to function as a metaplastic switch at the CA1-CA3 glutamatergic synapse. In order to accomplish this, it was necessary for us to revisit signalling cascades previously reported to regulate NMDAR function and plasticity using a consistently applied multidisciplinary experimental approach and common model systems.

Certainly past studies, including some of own, have described selected elements of both signalling cascades (i.e. those recruited downstream of PAC_1R and D1R). However, these findings are scattered across numerous studies using different experimental approaches to examine various endpoints (e.g. post-translational modification, protein trafficking or function) in numerous regions of the CNS. Just as an example, the study by Lei et al examines the contribution of D1R, PKA, Src family kinases and GluN2B to PCP-induced neurotoxicity. However, although the D1R-mediated enhancement of NMDAR function (i.e. recording of isolated NMDAR-EPSCs) is shown to involve PKA and GluN2B subunits, the contribution of Src family kinases, let alone Fyn, is not demonstrated. Inhibition of Src family kinases (by the non-selective inhibitor PP2) is shown to disrupt D1R-mediated neuroprotection, and, although D1R stimulation is shown to also cause increased GluN2B Tyr1472 tyrosine phosphorylation, causality between the two observations is not demonstrated. Lastly, while neuroprotection through enhanced GluN2B function is inferred, the consequence of selective GluN2A enhancement is not explored.

With regards to the study of synaptic plasticity, here again the specific contribution of NMDAR subunits, of selected signal transduction cascades and of Src family kinases to LTD and LTP have generally been explored individually rather than in parallel as we have done. Similarly, most studies of plasticity in the hippocampus narrowly focus on either LTD or LTP using standard induction protocols (e.g. 1 Hz, 900 sec and 100 Hz, 1 sec, respectively). Rarely have both been examined in a single study exploring physiological mechanisms that regulate plasticity, let alone has a range of induction frequencies been used in this context to characterize frequency-plasticity relations as we now report. As highlighted by Reviewer #2, while the specific contribution of GluN2A and GluN2B-containing receptors to the induction of various forms of synaptic plasticity has previously been examined, how the function of these two sub-populations of NMDARs is dynamically regulated by physiological factors such as GPCRs, thereby contributing to metaplasticity, remains poorly studied.

These differences highlight a significant strength of our studies, namely the side-by-side study of distinct signalling cascades that regulate NMDAR subunits using a consistent multidisciplinary experimental approach and model system. We feel quite strongly that collectively the findings reported in our study represent an important and highly original contribution to this area of study. Admittedly, our earlier draft did not go far enough in this respect. Accordingly, we have substantially revised the manuscript to address the reviewer's criticisms and now provide new data to address specific concerns regarding the quality and the interpretation of the experimental evidence provided. The additional data from GluN2AY1325F and GluN2BY1472F knockin animals (as described throughout this letter), is especially significant in this respect. Collectively these changes and additions to the manuscript have considerably improved the quality and impact of our findings and we hope that these changes will alleviate any remaining concerns held by the reviewer.

> *General comments:*
> *1. The paper needs some serious revision and clarification to make it easier to read and make the experiments understandable. Indeed, the experiments are not well described and important information such as the model and species used for each experiment as well as the concentrations of each substance used is missing. Especially, it is written in the material and methods that cell isolation has been performed from rats or mice but it is not known which species has been used in the different experiments.*
> *The duration of drug applications or how the drug is applied (bath solution, patch pipette...) is missing sometimes.*
> *Clearly, the way the experiments have been performed needs to be defined more precisely.*

Response to general comment 1

We apologize to the reviewer. The lack of experimental detail was a substantial problem in the previous version of the manuscript. The entire manuscript and the figures have been revised to ensure that all key information is clearly presented. The experiments are much more extensively described.

> 2. The order of the figures does not match with the order they are presented in the manuscript. Especially, figures 2c and d are described before fig 2a and b while it probably makes more sense to present the a and b before the c and d. Furthermore the Supplementary figure 2 should come before the supplemental figure 1 and the figure 2d to introduce Fyn(39-57) and Src(40-58).
We have corrected this as requested.

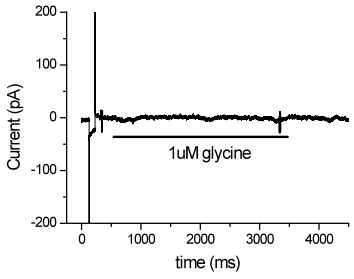
Supplementary fig. 6 should come after supplementary fig. 5 to be consistent with the order in the text.
We have corrected this and have altered the order and complement of figures in the manuscript.

> 3. The figures may be reorganised. Especially, the supplementary fig.3 and 4 and the Figures 4c and 4d could make a figure on its own since they all focus on synaptic plasticity.
We have corrected this and have altered the order and complement of figures in the manuscript.

New Figures 4 and 5 and 6 (figures 3 and 4 brought up from supplemental and old Fig 4 split in two
We have corrected this and have altered the order and complement of figures in the manuscript as requested by the reviewer.
>
> Specific comments:
> 1. Material and method is not complete: the rapid application system is not described, as well as the concentrations of NMDA and coagonist applied (In figure legend 1, the authors write that they doubled the concentration to have 100 µM NMDA and 1µM glycine, so we should deduce that they used 50 µM NMDA and 0.5 µM Glycine??). This raises the next question to understand why authors used glycine and not D-serine on neurons (Glycine is not specific to NMDAR and can activate other receptors).

Response to specific comment 1

The descriptions of the materials and methods with respect to drug applications has been extensively expanded and revised. In control experiments we have never seen any response of isolated CA1 hippocampal neurons to 1 mM glycine (over a period of ten years). Even much higher concentrations of glycine have been used (not by us) in cultured hippocampal neurons without activation of glycine receptors (Nong et al., 2003). Results from 5 neurons (mean -12 ± 2 pA before glycine; -15.4 ± 2.3 pA during glycine; -15.5 ± 3 pA, 3 seconds following glycine application). Essentially, these values are just the leak currents. See Letter Fig. 2.



Letter Fig. 2. Whole cell patch clamp of an isolated CA1 neuron. Currents are shown before, during and after an application of 1 mM glycine.

> 2. The concentrations of inhibitors such as NVP-AAM077, ifenprodil or PKI by example are not mentioned either while the selectivity of these compounds clearly depends on the concentration used.

Response to specific comment 2

We apologize for this oversight and have provided all the requested information. We chose concentrations that were appropriate for selectivity based upon the literature. NVP is only partially selective, even at the concentrations we employed (Neyton and Paoletti, 2006;Paoletti and Neyton, 2007). Zn (300 nM) is used at the concentration recommended by Pierre Paoletti (personal communication and used by Paoletti and collaborators to distinguish between GluN2A and GluN2B in the hippocampus (Nozaki et al., 2011)). Also, see response to comment #4 below.

> 3. It is difficult to understand why most of the experiments performed to identify the signaling pathways involved in NMDAR phosphorylation have been done with an intracellular medium

containing 140 mM CsF. Even if fluoride is known to improve quality of the patch recording, it can interfere with many signaling pathway, especially by inhibiting the phosphatases.
> 5. It is also surprising to see that only the peak NMDA current has been studied but not the steady state. In MacDonald et al, BBA 2007, authors wrote "Indeed we suspect that Ipeak is mediated in large part by receptors that only contain NR2A subunits (NR2AA, see below) (but not exclusively) whilst receptors containing only NR2B subunits (NR2BB) contribute more to the steady-state currents". Both parameters should then been analyzed.
> 6. The shape of the NMDA response (especially presence or not of a peak) is very different at t=0 min between all the NVP experiments (figure 1a, fig 1c, fig 2a and 2c). Could the authors discuss this or choose better example?

Responses to specific comments 3,5,6
Peak versus Steady-state currents, Shape of the currents and use of fluoride in some experiments but not others:

We have extensive experience recording from acutely isolated CA1 neurons (Lu et al., 1999;Lu et al., 2000;Huang et al., 2001;Kotecha et al., 2002;Kotecha et al., 2003;Macdonald et al., 2005;Xu et al., 2008;Yang et al., 2009). These cells possess large primary dendrites but much of the dendritic tree is lost. The advantage of these cells is that they come directly from the hippocampal slices of young adult rats as opposed to primary cultured neurons that come from foetal tissue and are grown over a period of weeks. We can be confident the isolated neurons are CA1 neurons as they are visually separated from the slices. In addition, they are lifted directly into the flow of the perfusion system permitting a rapid change of solution with complete perfusion of the entire cell (at the same time). We can reliably measure stable peak NMDAR currents over periods of over 30 minutes using this approach and apply various test drugs either in the perfusion solution or inside the patch pipette. This compares with neurons in situ in the slice where the exchange of solution is very much slower due to diffusion. Furthermore, in both slice and cultured neurons it is difficult if not impossible to ensure that the entire cell rapidly is exposed, uniformly to the agonist solution. Rapid and uniform application of agonists and antagonists is of great advantage in pharmacological studies of NMDARs.

Of course there are distinct disadvantages was well. For example, the population of NMDARs will include both residual synaptic and extrasynaptic receptors. Recordings from isolated cells using patch solutions containing fluoride are far more successful in retaining cell viability than those using non-fluoride solutions. These cells are physically more delicate than those recorded in situ. We have also previously shown that Src regulation of NMDARs is not disrupted in acutely isolated neurons by using control non-fluoride solutions as a comparison (Lu et al., 1999).

In parallel we were able to confirm the basic findings from isolated CA1 neurons for synaptic NMDAR mediated currents in the slice using whole cell patch clamp experiments using a non-fluoride solution (see methods). Furthermore, all of the neurochemistry was done in the absence of fluoride. Furthermore, by examining field potentials we were able to judge the population responses of these neurons without having to resort to whole cell recordings where signaling can be disrupted by the diffusional exchange between the patch pipette and the cell interior. We believe that we have been able to greatly strengthen the reliability of our results by using multiple recording techniques and by balancing weakness versus the strengths of each technique. Furthermore, we have also confirmed the neurochemical side of these changes in hippocampal slice that have been treated in parallel to our electrophysiological recordings.

“Shape of the currents” in acutely isolated CA1 neurons

Responses of acutely isolated CA1 neurons to rapid applications of NMDA are characterized by a peak response which decays to a quasi-steady state. Our applications of 2 seconds were chosen so that the peak could be readily identified. The peak was determined by visually setting the peak between two cursors. The program (pClamp) then determined the peak and averaged the largest 5 points. We have used this approach in a variety of previous publications (Lu et al., 1999;Huang et al., 2001;Kotecha et al., 2002;Kotecha et al., 2003;Macdonald et al., 2005;Beazely et al., 2008;Xu et al., 2008;Yang et al., 2009).
The apparent steady-state currents were never intended to be measured by us in this study. Firstly, the currents have not reached a true steady-state as the periods of application would have to be much longer to achieve this. Secondly, the quasi-steady state current is determined by the rates of desensitization of NMDARs. Desensitization is dependent upon at least three processes: 1) Conventional agonist-dependent desensitization 2) glycine-dependent desensitization 3) calcium-dependent desensitization (Lu et al., 1999;Lu et al., 2000;Lu et al., 2001) (Sather et al., 1990;Sather

et al., 1991;Sather et al., 1992;Johnson et al., 1992). The degree of desensitization of these currents varies substantially between individual cells, thus the relative size of the quasi-steady state currents to the peak is different in each neuron. The shape of the current therefore depends upon the individual cell recorded. In essence the peak currents more closely resemble the synaptic currents, as the quasi steady-state is unlikely to represent a physiological occurrence at the synapse. Therefore, we have measured peak currents and made direct comparisons between these peak currents and the behaviour of synaptic NMDAR currents. We have observed similar regulation of synaptic and peak currents is a variety of signal transduction studies completed over the last ten years (Lu et al., 1999;Huang et al., 2001;Kotecha et al., 2002;Kotecha et al., 2003;Macdonald et al., 2005;Beazely et al., 2008;Xu et al., 2008;Yang et al., 2009)

Further comments to comments 5 and 6

The kinetics of the GluN2A and GluN2B receptor whole cell responses are different but there is always considerable overlap. We speculated in the BBA article that with extremely fast and uniform applications of NMDA the involvement of NR2AA would be emphasized. However, in the vast majority of recordings the difference in kinetics of the receptors simply does not work as a means to separate the NR2AA and NR2BB components and indeed the peak, on average has roughly half and half contributions by these receptors.

The quasi-steady state currents reflects a complex state of desensitization of the various receptors subtypes and this added complexity confounds such measurments.

> 4. Also, the authors have used a concentration of zinc of 300 nM to block selectively NR2A component. However, 100 nM has been described to be nearly saturating at blocking NR1/NR2A (70 % inhibition, that is the maximum inhibition) with a low inhibition of NR1/NR2B. At 300 nM, NR1/NR2A is not more blocked, but NR1/NR2B is blocked at more than 30%. The authors should then reconsider the experiments

Responses to specific comment 4

We used the concentration recommended to us by Pierre Paoletti (personal communication) who has extensively characterized the Zn block of subtypes of NMDARs. He has specifically used this concentration in the hippocampus to maximally block GluN2AR over GluN2BR currents (Nozaki et al., 2011). We agree that, as is the case for NVP the selectivity is not ideal. Given the concerns about pharmacological specificity we chose to design three different experiments: 1) selectivity of block by low concentrations of NVP, which should partly distinguish between GluNR2A and GluNR2B 2) selectivity by block of a concentration of Zn that will give maximal inhibition of GluNR2A (although not complete) with some effect on GluNR2B 3) selectivity of the signaling pathways in GluN2A-/- mice that are entirely independent of antagonists. The use of the knockouts is likely the strongest support for our interpretation. We would argue that by using three different experimental approaches we have reached the appropriate interpretation.

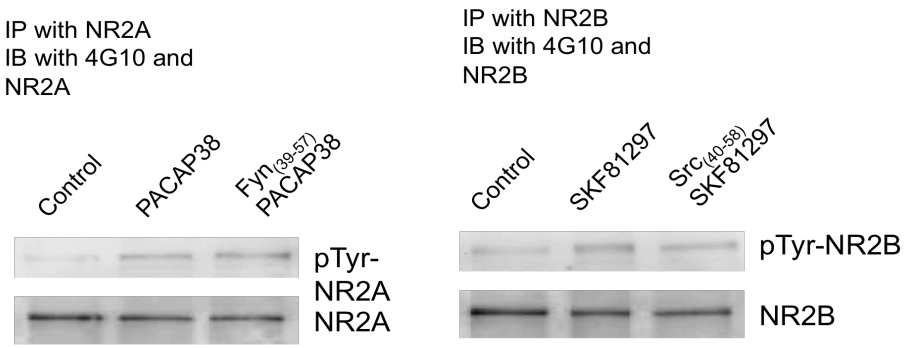
> 7. The authors have also designed a new inhibitor to block Fyn kinase activity specifically, but no proof of the specificity of this inhibitor is given.

Responses to specific comment 7

We have clarified the manuscript. This Fyn peptide and Src(40-58) are **not kinase** inhibitors. They presumably prevent the kinases from gaining appropriate access to the receptors for phosphorylation. The selectivity is entirely in terms of the relative abilities of Src versus Fyn to phosphorylate the NMDARs and regulate their function. The interfering Fyn peptide does not block the potentiation induced by Src (or PACAP) and the interfering Src peptide does not block the effect of recombinant Fyn (or SKF). The selectivity is further illustrated in the new Supplemental Fig. 1.

> 8. To ascertain that each receptor effect is due to specific kinase activation, the authors have tested the level of tyrosine phosphorylation for GluN2A and GluN2B without GPCR activation, with GPCR agonist and with GPCR agonist and a kinase inhibitor. But why have the authors only tested the inhibitor they expected to have an effect for each GPCR agonist? In each case we would have expected to see both inhibitors (Fyn and Src). Moreover as PKA has a clear role in D1R induced potentiation of NMDA response, its lack of effect at least on GluN2B tyrosine phosphorylation could have been controlled.

We have clearly shown in Figure 3 (and supplemental Fig. 1) that the activation of Src is prevented by Src(40-58) but not by Fyn (39-57). In addition we showed that the activation of Fyn is blocked by Fyn(39-57) and not by Src(40-58). This clearly demonstrates the selectivity of kinase activation. Furthermore, our new experiments on knockin mice show; firstly, that the PACAP changes in NMDAR currents and plasticity depend directly upon phosphorylation of GluN2AR. Secondly, SKF enhancement of NMDAR currents and plasticity directly depend upon phosphorylation of GluN2BR. We would argue that this more than sufficient to establish the GPCR signaling and targeting of receptor subtypes. We also performed the negative control experiments requested: Fyn(39-57) does not block the tyrosine phosphorylation of GluN2A by PACAP and Src(40-58) does not block the tyrosine phosphorylation of GluN2B by SKF (see Letter Fig. 3 below).



Letter Fig. 3. Hippocampal slices were pretreated with the inhibitory peptide Fyn or Src (10 μM, each; TAT conjugated forms) for 30 min in oxygenated-aCSF, then exposed to PACAP38 (1 nM) or SKF81297 (10 μM) for 20 min. The slices were thoroughly washed 3 times with cold PBS before being lysed for IP and WB analysis. Phosphotyrosine signal intensities (pTyr-NR2A or pTyr-NR2B) were normalized to the expression intensity of NR2A and NR2B, respectively. Normalized values are as follows: control 7.7, PACAP38 12.7, PACAP + Fyn(39-57) 14.1; control 6.6, SKF81297 14.6, SKF + Src(40-58) 14.6

We have shown that D1R activation leads to enhanced currents and these effects are blocked by inhibitors of PKA (see new Fig. 2D, PKI; and, Rp-cAMPS (not shown). It was previously shown that PKA activation leads to enhanced tyrosine phosphorylation of GluN2B and enhanced CA1 synaptic currents via stimulation of Fyn kinase (Yaka et al., 2003).

> 9. All the synaptic plasticity data have to be reanalyzed. Synaptic plasticity has been evaluated through the measure of the amplitude of the EPSP and never the slope. This parameter should not be used as amplitude can be contaminated by population spikes (Such a contamination can be distinguished in the traces illustrated).

Responses to specific comment 9
We have reanalysed all of the data as requested. Only slopes are now employed.

> 10. It is not described in this manuscript how the values of the bar graphs presented in figure 1, 2 and 4 have been calculated. For several experiments, a plateau (and so the maximal value) is not reached within the duration of the experiment (Fig 1a (Src + Ro25-6981); figure 2a (Fyn + Ro25-6981); figure 2a (Fyn + NVP-AAM077); figure 2c...). If the authors have chosen an intermediate time before the maximal amplitude of the effect is reached, they should mention this time and discuss their choice.

Responses to specific comment 10
The duration of the patch clamp recordings is the primary determinant of when responses were measured. These are difficult electrophysiological recordings requiring a high degree of skill. It is very difficult to record for periods much greater than 30 mins and given the large number of required recordings we focused on values between 20 and 30 mins. We do not claim that there is a maximal value and we are only looking at changes relative to controls.

We examined averaged responses between 20 and 30 mins (usually between 20 and 25 minutes following bath application of an agonist or 25 to 30 mins following breakthrough when using kinases in the patch pipettes). This information is now explicitly stated in the figure legends.

> 11. In the EPSC experiments (figure 4), some data with a GluN2A antagonist (NVP-AAM077) on figure 4a could be of interest to show that the increase on EPSC is mediated via this subunit in this model as well..Applying an NR2A or NR2B antagonist, at least on 1 of the stimulation (10 Hz by example), could also be of interest for the figure 4c-d to show that the effect on metaplasticity is mediated via these independent subunits.

We appreciate the reviewer's suggestion for an additional experiment. However, NVP is at best selective by only one order of magnitude (must be used at concentrations of 40 to 50 nM). Furthermore, NVP should not be used in studies of synaptic currents primarily because the affinity of glutamate for NR2A versus NR2B differs dramatically (see (Neyton et al., 2006;Paoletti et al., 2007) for detailed explanation). It can be used to distinguish receptor subtypes on isolated neurons where the concentrations of NMDA are near the EC_{50} values and because the affinity of NMDA for NR2A versus NR2B is very similar (Ishii et al., 1993;Wyllie et al., 1996). In any case, we believe that the new experiments with knockin mice provide much stronger support than could be achieved by the continuation of this pharmacological approach.

> 12. Could authors comment the recent article from Lei et al reporting "that activation of dopamine D1 receptors (D1R) with dihydrexidine (DHX) increases serine phosphorylation of the NR1 subunit through protein kinase A activation and tyrosine phosphorylation of the NR2B subunit via Src kinase. DHX also facilitated the synaptic response in cortical slices and this was blocked by an NR2B antagonist."
>

The findings in the article by Lei, et al largely support the salient properties described in a subset of our own. Specifically, in PFC cultured neurons, the authors demonstrate increased NR2B tyrosine phosphorylation (Tyr1472) by a Src family kinase member in response to D1R stimulation in PFC cultures. They also show that D1R stimulation enhanced NMDR-mediated synaptic responses (NMDAR-EPSCs). The enhancement observed was entirely dependent on the NR2B-containing synaptic NMDARs.

Beyond these superficial similarities, the two studies, each with their own merit, are really quite distinct and aim to answer very different questions. Theirs examines the mechanisms responsible for the observed reduction in PCP-induced neurotoxicity by D1Rs, and is focused primarily on examining the specific contribution made by NR2B-containing NMDARs. In addition, save for one figure examining the function of NMDARs directly (Fig 5a-d), the Lei et al study is primarily concerned with cell death as an end point to examine the consequence of altered NMDAR regulation (biochemical approaches used as a complement). It is worth noting that the contribution of Src family kinases to the enhancement of NMDAR-EPSCs was not examined in their study (a PKA inhibitor was tested but not for example PP2). In contrast, ours demonstrates that neuromodulatory transmitters, acting upon their cognate GPCRs, can recruit distinct signaling cascades to alter the function of specific NMDAR subpopulations thereby providing a molecular mechanism for metaplasticity. The focus here has been to use biochemical techniques to support direct functional evidence that NMDAR subtypes are selectively regulated by GPCRs through functionally segregated intracellular signalling cascades. We utilize inhibitors capable of discriminating between Src and Fyn kinases and show that these specific Src family kinase members are **differentially** recruited by PAC_1R and D1R, respectively. Indeed, one limitation of the Lei et al study is that they could not distinguish amongst Src family kinase members since the inhibitors used are not selective.

Apparent difference between the two studies in the reported contribution of PKA to D1R-dependent regulation of NMDARs is also worth considering. The Lei et al study reports that D1R stimulation recruits PKA, leading to increased NR1-Ser897 phosphorylation. They also demonstrate that PKA inhibition abrogates neuroprotection against PCP-induced cell death by D1R stimulation. However, a causal relationship between increased NR1 phosphorylation and neuroprotection was not shown. More relevant when comparing our two studies, although the Lei et al study demonstrates that a PKA inhibitor could prevent D1R-mediated enhancement of NMDAR-EPSCs, they did not determine whether NR1-Ser897 phosphorylation was specifically required and more importantly, whether PKA-dependent regulation of NMDAR function required a Src family kinase (i.e. whether Src family kinase is downstream of PKA). We have previously reported on

such a scheme whereby Gαq-coupled GPCRs (e.g. mAchR) regulate NMDARs through a sequential signalling cascade involving PKC and requiring downstream Src-mediated tyrosine phosphorylation of NMDARs (Lu et al., 1999).

Although, we did not specifically examine NR1 serine/threonine phosphorylation, we find that the functional regulation of GluN2B-containing NMDARs by D1R stimulation can be entirely attributed to Fyn tyrosine kinase activation (i.e. prevented in the presence of Fyn(39-57)). More conclusively, we now show that D1R stimulation is without functional effect when Tyr1472 of GluN2B is replaced with phenylalanine. We also show that D1R regulation of plasticity at a frequency (10 Hz) corresponding approximately to theta (transition point from LTD to LTP) is absent in slices from GluN2BY1472F knockin mice. Accordingly, although NR1 phosphorylation may perhaps be altered, the functional consequence of D1R stimulation can be entirely accounted for by increased Fyn-mediated GluN2B-Tyr1472 phosphorylation, at least in hippocampal CA1 pyramidal neurons. Identifying the functional consequence of NR1-Ser897 phosphorylation is beyond the scope of the present study, but worthy of future consideration.

> Many minor points also have to be corrected. For examples:
> - the text is not uniform (size of police, indentation, etc., especially in the mat and meth section).
> - the different panel in each figure are not uniform (bar graphs, ticks, thickness of lines, police size...).
> - the reference to the supplementary data is not consistent (Fig S2A or Supplemenary figure 2A, by example).
> - For the paired-pulse facilitation, the supplementary fig.5b is put in reference instead of the supplementary fig.6.
> - In the material and methods the authors give the ref 21 (twice and not in good format) while this ref 21 is not the good one.
> - The reference list should not contain numbers.
> - A space should be added before each ref in the text.
> - There is a mistake in the y-axis labelling in fig 4c.
> - figure 4a and 4b1: no scale bar for the traces, illustrated
> - figure 4d scale bar only for the two upper traces nor the two lower.
>

The manuscript has been extensively revised to make these corrections.

REFERENCES

1. Beazely MA, Lim A, Li H, Trepanier C, Chen X, Sidhu BR, and MacDonald JF (2008) Platelet-derived growth factor selectively inhibits NR2B-containing NMDA receptors in ca1 hippocampal neurons. *J. Biol. Chem.*.

2. Huang YQ, Lu WY, Ali DW, Pelkey KA, Pitcher GM, Lu YM, Aoto H, Roder JC, Sasaki T, Salter MW, and MacDonald JF (2001) CAK beta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron,* **29,** 485-496.

3. Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, and Masu M (1993) Molecular characterization of the family of the N-methyl-D- aspartate receptor subunits. *J. Biol. Chem.,* **268,** 2836-2843.

4. Johnson JW and Ascher P (1992) Equilibrium and kinetic study of glycine action on the *N*-methyl-D-aspartate receptor in cultured mouse brain neurons. *J. Physiol. (Lond.),* **455,** 339-365.

5. Kotecha SA, Jackson MF, Al Mahrouki A, Roder JC, Orser BA, and MacDonald JF (2003) Co-stimulation of mGluR5 and N-methyl-D-aspartate receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons. *J Biol Chem,* **278,** 27742-27749.

6. Kotecha SA, Oak JN, Jackson MF, Perez Y, Orser BA, Van Tol HHM, and MacDonald JF (2002) A D2 class dopamine receptor transactivates a receptor tyrosine kinase to inhibit NMDA receptor transmission. *Neuron,* **35,** 1111-1122.

7. Lu W, Man H, Ju W, Trimble WS, MacDonald JF, and Wang YT (2001) Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron,* **29,** 243-254.

8. Lu WY, Jackson MF, Bai D, Orser BA, and MacDonald JF (2000) In CA1 pyramidal neurons of the hippocampus protein kinase C regulates calcium-dependent inactivation of NMDA receptors. *J. Neurosci.,* **20,** 4452-4461.

9. Lu WY, Xiong ZG, lei S, Orser BA, Dudek E, Browning MD, and MacDonald JF (1999) G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. *Nat. Neurosci.,* **2,** 331-338.

10. Macdonald DS, Weerapura M, Beazely MA, Martin L, Czerwinski W, Roder JC, Orser BA, and MacDonald JF (2005) Modulation of NMDA receptors by pituitary adenylate cyclase activating peptide in CA1 neurons requires Galphaq, protein kinase C, and activation of Src. *J. Neurosci.,* **25,** 11374-11384.

11. Martin LJ, Zurek AA, MacDonald JF, Roder JC, Jackson MF, and Orser BA (2010) Alpha5GABAA receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. *J Neurosci.,* **30,** 5269-5282.

12. Neyton J and Paoletti P (2006) Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. *J. Neurosci.,* **26,** 1331-1333.

13. Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT, and Salter MW (2003) Glycine binding primes NMDA receptor internalization. *Nature,* **422,** 302-307.

14. Nozaki C, Vergnano AM, Filliol D, Ouagazzal AM, Le GA, Carvalho S, Reiss D, Gaveriaux-Ruff C, Neyton J, Paoletti P, and Kieffer BL (2011) Zinc alleviates pain through high-affinity binding to the NMDA receptor NR2A subunit. *Nat. Neurosci.,* **14,** 1017-1022.

15. Paoletti P and Neyton J (2007) NMDA receptor subunits: function and pharmacology. *Curr. Opin. Pharmacol.,* **7,** 39-47.

16. Sather W, Dieudonne S, MacDonald JF, and Ascher P (1992) Activation and desensitization of N-methyl-D-aspartate receptors in nucleated outside-out patches from mouse neurones. *J. Physiol. (Lond),* **450:643-672,** 643-672.

17. Sather W, Johnson JW, Henderson G, and Ascher P (1990) Glycine-insensitive desensitization of NMDA responses in cultured mouse embryonic neurons. *Neuron,* **4,** 725-731.

18. Sather WA, MacDonald JF, and Ascher P (1991) Time-dependent changes in the N-methyl-D-aspartate receptor desensitization. In *Transmitter amino acid receptors: structures, transduction and models for drug development*, Barnard,E.A. and Cose,E. (eds) . Thieme Medical Publishers, New York, pp. 493-502.

19. Wyllie DJ, Behe P, Nassar M, Schoepfer R, and Colquhoun D (1996) Single-channel currents from recombinant NMDA NR1a/NR2D receptors expressed in Xenopus oocytes. *Proc. R. Soc. Lond. B. Biol. Sci.,* **263,** 1079-1086.

20. Xu J, Weerapura M, Ali MK, Jackson MF, Li H, Lei G, Xue S, Kwan CL, Manolson MF, Yang K, MacDonald JF, and Yu XM (2008) Control of excitatory synaptic transmission by C-terminal Src kinase. *J. Biol. Chem.,* **283,** 17503-17514.

21. Yaka R, He DY, Phamluong K, and Ron D (2003) Pituitary adenylate cyclase-activating polypeptide (PACAP(1-38)) enhances N-methyl-D-aspartate receptor function and brain-derived neurotrophic factor expression via RACK1. *J Biol Chem,* **278,** 9630-9638.

22. Yang K, Trepanier CH, Li H, Beazely MA, Lerner EA, Jackson MF, and MacDonald JF (2009) Vasoactive intestinal peptide acts via multiple signal pathways to regulate hippocampal NMDA receptors and synaptic transmission. *Hippocampus*.

2nd Editorial Decision 12 October 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your manuscript has now been seen by the original referees # 1 and 2, and their comments are provided below. As you can see, both referees appreciate that added changes and support publication in the EMBO Journal. I am therefore pleased to proceed with the acceptance of the paper here. Before doing so there is a minor remaining issue, referee #1, that I would appreciate if you would respond to in a final revision. As soon as we receive the revised version, we will proceed with its acceptance here.

Thank you for submitting your interesting manuscript to the EMBO Journal

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The authors have done extensive revision, including carefully designed and executed new experiments on NMDA R knock in mouse mutants, to substantiate their conclusions, as well as extensive rewriting of the text and detailed responses to my questions.
My only question relates to the knock in experiments. They show that "PACAP38 and SKF81297 failed to potentiate NMDAR-induced currents in cells from GluN2A(Y1325F) and GluN2B(Y1472F) mice, respectively", but they do not show whether PACAP and SKF were able to potentiate GluN2B(Y1472F) and GluN2A(Y1325F) mice, respectively (ie the converse experiment to show specificity of the required phospho-site). If possible this should be included or stated.

Referee #2:

The revised ms has adequately addressed most of concerns I raised during the first round of reviewing process. In particular, the new data from the two knock-in mice have further strengthened authors' case. In my opinion, it can now be recommended for publication in the journal.

2nd Revision - authors' response 08 November 2011

We have done some additional experiments, as requested by the reviewer. These results are now given at the end of page 11 and on to page 12:

"Furthermore, PACAP38 (1nM) increased NMDAR-mediated currents in isolated neurons from GluN2B(Y1472F) (n=5 cells, 1.4 +/- 0.14) mice and SKF 81297 (10 μM) increased those taken from GluN2A(Y1325F) (n=4, 1.2 +/- 0.02)."

