Neuropeptide Y regulates the hematopoietic stem cell microenvironment and prevents nerve injury in the bone marrow

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Editor: Thomas Schwarz-Romond

1st Editorial Decision 30 October 2014

Thank you very much for sending your results for in-depth consideration to The EMBO Journal editorial office.
The study has now been assessed by three expert referees with their critical remarks being attached below.

As you will recognize, all three recognize the value of the truly impressive amount of presented data. They also emphasize their timeliness, certain level of novelty as well as potential clinical relevance.

Irrespective of these rather encouraging comments, it becomes obvious that the study aims in fact to address too many issues at once and thus becomes, at least in part, too descriptive. I very constructive suggestion that I more than happy to convey, would therefore be to split the data into two manuscripts as to focus individually on (i) the basic biological part and the (ii) potential clinical applications arising from this.
One humble suggestion would be to integrate current figures 1,2,3,6 maybe 9 and 5(9), 6, 7, 4, 8 into such papers, respectively.

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It has to be said however that a simple recast would not suffice. Separating the basic from the applied part should encourage you to experimentally go further into potential molecular mechanisms on how NPY would elicit its regulatory effects within the HSC/niche homeostasis:

- please refer specifically to ref#1 points 2, 3;
- relevant critiques from ref#2 (Lyz2-cre specificity etc.)
- truly valuable suggestions from ref#3 on molecular underpinnings (particularly experimental points 1, 2, 3, 4, 6)

Please be assured that I consulted with two of the referees on this possibly unusual approach as to warrant a fair subsequent assessment of the resulting complementary papers.

Conditioned on this, I would be delighted to engage/hear your opinion on anticipated timeline and feasibility of the suggested further reaching experiments.

I hope that this proposal meets your approval and may eventually increase focus, general interest and visibility of your highly interesting research findings.

Yours truly,

Thomas

P.S. Please use the link for submission of one of the potential two future studies while co-submitting a related one afresh, marking/informing me via direct E-mail at the point of co-submission.

REFEREE REPORTS:

Referee #1:

The paper by Park MH et al importantly shows for the first time that mice deficient in Neuropeptide Y (NPY) exhibit alterations in the survival, function and traffic of hematopoietic progenitors. Overall, the authors have performed an extensive characterization of the bone and marrow of these mice. Notably, the authors have deleted NPY receptor in candidate stromal hematopoietic (macrophages) and non-hematopoietic (osteoblasts) cells and attribute specific effects on HSCs to NPY signaling in these populations. However, some cause-effects relationships withdrawn are not supported by experimental evidence. For instance, the authors assume that NPY is only produced by sympathetic nerve fibers and that the loss of autocrine NPY signaling in these neurons and other effects on endothelial cells account for the reduction in bone marrow HSCs, but this is not demonstrated. Other studies have shown that NPY is produced by macrophages and other bone marrow-derived cells and thus suggest other possibilities. The authors do not provide an explanation as why and how some stromal populations are responsible for specific effects on the hematopoietic and skeletal phenotype. Below are specific comments aimed to improve this paper.

1. Figure 1J shows around 40% lethality of control mice that received two split doses of 5 Gy and that were mice transplanted afterwards with 1 million nucleated cells from congenic mice. Why is there such lethality in mice that should be successfully reconstituted? Are NPY KO mice in a pure C57BL/6 background?

2. The authors assume that NPY is produced by sympathetic nerve fibers, but a recent report has shown that NPY is abundantly produced by bone marrow macrophages and that its production by hematopoietic cells is involved in obesity-induced inflammation (Singer K et al. PLOS One 2013). The authors should test the effect of NPY deficiency in the hematopoietic system (e.g. control mice transplanted competitively with control CD45.1 and KO cells).

3. Along the same line, the authors interpret that NPY produced by sympathetic nerve fibers has autocrine survival effects on these neurons and has also protective effects on endothelial cells. They assume that both of them would contribute to preserve HSC function. However the cause-effects relationships remain unclear, as whether these fibers are indeed the main source of NPY in the bone marrow. There is no demonstration of the claim in Figure 2 Legend title. Also, in the Discussion
P.22 L.10: destruction of SNS fibers caused a decrease in the number of ECs, (also nestin+ cells?)...some cause-effect relationships are withdrawn without much supportive evidence.

4. Figure 2C shows a significant increase in apoptotic cells in the bone marrow of NPY KO mice. What are these cells? How do the authors reconcile increased apoptosis of bone marrow cells, increased quiescence of hematopoietic progenitors and yet no change in bone marrow cellularity?

5. The authors show very specific effects of NPY signaling in macrophages and osteoblasts on HSC survival/function and trafficking, respectively. How does Y1 receptor activation on macrophages (and not in osteoblasts) exert this specific function on HSCs?

6. How do the authors explain the apparently contradictory results in NPY KO mice that show increased SDF-1, Angpt1 and Kitl BM mRNA expression levels but no difference in HSPC homing?

7. The NPY dose injected in mice seems higher than the physiological range, questioning whether some responses would be expected to occur normally in vivo.

8. How do NPY receptor expression levels compare between osteoblasts, endothelial cells and nestin+ cells? Can the different response of these cells to NPY be explained by different expression of the receptor?

9. Figure 1 Legend title seems wrong.

10. The recovery of ovariectomy-induced osteoporosis by NPY is remarkable. It was not clear to me how the mobilization of osteoclast precursors was studied and how it might contribute to explain the phenotype. It is very unlikely that some mobilization of HSCs to peripheral circulation would account for reduced osteoclast differentiation.

Minor comments:

1. Some macrophages in the bone marrow have been associated with osteoblasts and some have been shown to regulate HSC traffic. Even though Lyz2-cre-driven excision of NPY receptor does not seem to affect HSC traffic, have the authors noted differences in endosteal macrophages?

2. P. 19, 1st para, L. 3-4: that some HSC maintenance genes show higher expression does not imply higher HSPC number, and actually the data in Fig. 8C does not show any difference.

Referee #2:

The authors investigate the role of NPY on hematopoietic dynamics with extensive analysis of gain and loss of function models. They show that NPY leads to HSC egress from the bone marrow and, conversely, deletion of the NPY receptor is protective. These effects are mediated by the expression of known HSC niche molecular components, such as SDF1, angiopoietin 1 and Kit ligand.

Overall the data are extensive and the work is novel. The biggest issue for the reader is to find the right way through the mass of data, with the explanation of the experiments being not always as clear as it could be (some examples below). Rewriting completely the manuscript with a more clear focus and more succinct figures will greatly increase the readability and will communicate a more clear message.

There are however some considerable concerns that the authors should address/rectify.

One considerable oddity is the low level of engraftment observed in all transplantation experiments described (Figure 4L-N, 10^6 BMNC should give rise to 95% of chimerism, not 1.5%; Figure 5 L-N, a 1:1 competitive transplant should lead to 50% chimerism, not 10%).

It is also not clear how Lyz2-cre would target only perivascular macrophages but not, for example, osteoblasts localized near osteoblasts. either this point is proved or the description of the mouse strain and the conclusions made should be changed.
In Figure 1O it seems the colonies are 95% of host origin, hence showing no significant difference. Colony assays following better engraftment would be more informative.

Egress of HSCs in response to mobilizing agents should be explored too as NPY will likely affect this process. LPS is an interesting reagent to study and an excellent model of systemic stress, but not a routinely used mobilizing agent.

Figure 2C: it is not clear what BM cells were assessed for apoptosis.

Figure 4E: NPY treatment recover chemotherapy-induced HSC impairment only in Col cre mice, not in all strains.

Figure 2G-J: in vitro cultures of cell lines are an experimental model that is very far from in vivo neurons. This is an example of data that does not majorly strengthen the manuscript and could be moved to a supplement.

Figure 7A-C is largely a repetition of previous data and could be shrunk considerably.

The ovariectomized model is very complex and not fully characterised. Osteoclasts numbers could be analyzed also independently of ovariectomy.

The data presented in Figure 5 and 9 do not seem to agree. NPY +/- mice should have increased niche factors already at baseline and not in response to Y-mediated stress response.

Overall the language should be improved. Just an example:
Page 10. 'Did not affect SNS fiber expression': fibers cannot be expressed.

Referee #3:

In this interesting study the authors show the involvement of NYP and its receptor in HSC repopulation, maintenance and mobilization. They look at potential effects of NYP on macrophages, osteoclasts, nerve and endothelial cells. By using different transgenic mouse models they show that NPY deficiency causes BM impairments of HSC survival and mobilization, as well as integrity of SNS nerve fibers. Pharmacological or endogenous NPY increase leads to HSC regeneration and mobilization through the Y1 receptor. In addition, NPY treatment prevents cisplatin-induced deficits in BM function and ovariectomy-induced bone loss in mice through the Y1 receptor. Although, the authors use a lot of assays, murine models and tools as well as show everything in several ways, no novel mechanistic insights are shown in the manuscript. The only suggested regulator is p53 which is shown only in a correlative way. Dependency on p53 is not proved by the data. In addition, the manuscript is too loaded with data and difficult to follow. Therefore, I suggest that the authors should choose and focus on a specific message and re-edit the manuscript accordingly. For example, the manuscript may be divided into two different papers: one dealing with mobilization and the other with HSC maintenance and stress-induced response. There are too many results and the authors are encouraged to consider splitting the MS into 2 studies, 1 focused on stem cell maintenance and mobilization and the other on bone and bone marrow integrity and how to overcome osteoporosis.

Major comments
1. In their results the authors observed that NPY +/- mice exhibit reduced numbers of HSPC in the BM and in the PB, in addition to reduced numbers of niche elements such as Sca-1+ MSPC. The conclusion made by the authors that the reduced numbers of HSPC in the PB is due to reduced HSPC egress might be wrong as reduced numbers of HSPC in the PB may be derived from their reduced numbers in the BM. Assays to examine BM HSPC motility and migration capacity should be performed to enlighten this aspect and help to make a final conclusion.

2. The authors examined HSPC recovery after 5-FU in dependency of NPY expression. 5-FU treatment induces mobilization by itself in a CXCL12/CXCR4 dependent manner, involving FGFR1
signaling (as shown by Meng et al., Blood 2012 (please cite)). What is the response of NPY-/- mice to 5-FU induced mobilization and how are FGFR1/CXCL12/CXCR4 elements and function are altered in these mice?

3. The authors provide evidence that NPY deficiency results with enhanced apoptosis of BM cells and conclude that apoptosis of niche elements are leading to reduced HSPC numbers. However this may be derived from total apoptosis effects which are general for all BM cells including HSPC and is not only dependent on niche function and availability. How can the authors conclude that the reduced HSPC numbers is not due to cell-autonomous effect in which NPY deficiency leads to increased apoptosis among HSPC? Please test direct effects of NPY on enriched HSPC in vitro.

4. The authors introduce macrophages as part of the affected niche in their study. Yet, the markers defining macrophages that are used by the authors actually label a broad myeloid population. Recently a very small population of alphaSMA expressing macrophages was shown to maintain BM HSPC under steady state and during stress induced conditions such as myeloablative irradiation treatment which requires BM recovery (Ludin et al., Nature Immunology 2013 (please cite)). What is the status of these cells under NPY deficiency of when total myeloid Lyz2-Cre system is activated to delete NPY receptor? What is their contributions to HSPC egress and mobilization as HSPC anchoring cells in the BM? What is the role of this population in nerve fiber recovery?

5. How come there is enhanced expression of HSC maintenance factors in the BM of NPY-/- mice yet their BM numbers, and maintenance is reduced? Who provides these factors in enhanced manner if there is enhanced apoptosis of different niche elements? Please explain this contradiction.

6. The authors here used LPS-induced mobilization, but did not relate it to their previous findings. Please test the effect of G-CSF as well as AMD3100-induced mobilization in NYP deficient mice. Also, what will happen to HSPC egress when G-CSF or AMD3100 will be administrated with NPY antagonist? The authors show that NPY injection induced rapid HSPC mobilization already after 1h. This mobilization was accompanied with reduced levels of CXCL12 in the BM. What about CXCL12 levels in the PB? It was recently shown that rapid mobilization involves CXCL12 secretion from the BM into the PB (Dar et al., Leukemia 2011(please cite)). Is it the same case with NPY-induced mobilization? What is the status of CXCR4 expression and CXCL12 induced migration of HSPC following NPY injection? Please test in vitro migration and surface CXCR4 expression levels.

7. The effect of NPY in osteoblasts that enables to escape BM failure following chemotherapy is shown to be mediated by NPY1R. Which signaling pathway does this involve?

8. The authors should go deeper in the mechanistic pathways of NYP regulation: how it affects HSPC mobilization (both directly and indirectly via the microenvironment, how it regulates bone-remodeling, etc.

9. What is the physiological role of NPY and how it affects HSC and osteoblasts? A direct in vitro assay should be done with sorted HSC and osteoblasts.

10. More data should be added on the mechanism or the factors from the macrophages that mediate neural protection in BM niche.

Minor comments
1. The authors should avoid statements like "for the first time" and also should be more modest about their conclusions.

2. The authors over-claim that NPY might be a treatment for osteoporosis. There were no human subjects in this work with osteoporosis that were given NPY.

3. In figure 5.D, the authors can add "NPY" to the graph of Osteoblast differentiated from BM-MSC, to make it clearer.

4. The figures are very crowded with a lot of data and many graphs therefore, it is very difficult to
follow them. More clarity and less fig. are encouraged, in line with our suggestion to split the data into 2 MS.

Referee#1:
The paper by Park MH et al importantly shows for the first time that mice deficient in Neuropeptide Y (NPY) exhibit alterations in the survival, function and traffic of hematopoietic progenitors. Overall, the authors have performed an extensive characterization of the bone and marrow of these mice. Notably, the authors have deleted NPY receptor in candidate stromal hematopoietic (macrophages) and non-hematopoietic (osteoblasts) cells and attribute specific effects on HSCs to NPY signaling in these populations. However, some cause-effects relationships withdrawn are not supported by experimental evidence. For instance, the authors assume that NPY is only produced by sympathetic nerve fibers and that the loss of autocrine NPY signaling in these neurons and other effects on endothelial cells account for the reduction in bone marrow HSCs, but this is not demonstrated. Other studies have shown that NPY is produced by macrophages and other bone marrow-derived cells and thus suggest other possibilities. The authors do not provide an explanation as why and how some stromal populations are responsible for specific effects on the hematopoietic and skeletal phenotype. Below are specific comments aimed to improve this paper.

We would like to thank the referee for these comments concerning our manuscript. According to the other referee’s comment, we have separated the data into two manuscripts to focus on a more specific message and readability.

Manuscript 1

demonstrates HSC impairment due to NPY deficiency and the potential therapeutic application of NPY in chemotherapy-induced bone marrow nerve injury.

“Response for manuscript 1”

1. Figure 1J shows around 40% lethality of control mice that received two split doses of 5 Gy and that were mice transplanted afterwards with 1 million nucleated cells from congenic mice. Why is there such lethality in mice that should be successfully reconstituted? Are NPY KO mice in a pure C57BL/6 background?

The reviewer is correct that we found that control mice receiving two split doses of 5 Gy showed around 40% lethality, although mice were transplanted 10⁶ bone marrow (BM) nuclear cells from CD45.1 mice (Figure 2A in revised manuscript 1). To confirm successful reconstitution of transplanted CD45.1+ BM cells in control recipient mice, we analyzed the percentage of transplanted CD45.1+ cells in BM of control recipient mice through flow cytometry. Additional Figure 1 (below) shows 86.48% of CD45.1+ cells in BM of control recipient mice. Therefore, we conclude that the reduced survival of control recipient mice is due to different strains of mice. Actually, the strain of control mice used in Figures 1-5 of revised manuscript 1 is 129S1/SvImJ because the NPY−/− mice’s background is 129S1/SvImJ. Figure 7B in revised manuscript 1 shows 100% survival of PBS injected mice (C57BL/6 background), supporting this reason.

Additional Figure. 1. Flow cytometry plot in BM of control recipient mice. CD45.1+ cells showed 86.48% in BM of control recipient mice.

“Response for manuscript 1”

2. The authors assume that NPY is produced by sympathetic nerve fibers, but a recent report has shown that NPY is abundantly produced by bone marrow macrophages and that its production by
hematopoietic cells is involved in obesity-induced inflammation (Singer K et al. PLOS One 2013). The authors should test the effect of NPY deficiency in the hematopoietic system (e.g. control mice transplanted competitively with control CD45.1 and KO cells).

We agree with the referee’s opinion. It is noted that NPY is produced by sympathetic nerve fibers, as well as bone marrow hematopoietic cells (J Exp Med. 2005, 202:1527-1538; Peptides. 2007, 28:435-440).

As suggested by this referee’s comment, we carefully investigated whether NPY depleted bone marrow nuclear cells (NPY KO BMNCs) contributed to HSC engraftment and regeneration in the marrow niche. We transplanted $1 \times 10^6$ WT CD45.2$^*$ or NPY KO BMNCs into lethally irradiated WT mice. The results showed no significant difference in mice survival, repopulating efficiency and the percentage of LSK cells in BM of each group at 8 weeks after transplantation (Figures. 2J-L in revised manuscript 1). Moreover, we performed a homing assay in BM at 24 hour after transplantation. NPY KO BMNCs did not affect hematopoietic progenitors homing to the BM (Figure. 2M in revised manuscript 1). These results indicated that NPY deficiency in bone marrow cells did not affect bone marrow regeneration. Therefore, we can suggest that NPY produced by sympathetic nerve fibers was required for bone marrow regeneration. In revised manuscript 1 we have added these results to better convey our message as Figure. 2J-M.

![Figure. 2J-M in revised manuscript 1. NPY deficiency in bone marrow cells does not affect bone marrow regeneration. Lethally irradiated WT mice were transplanted by $1 \times 10^6$ WT CD45.2$^*$ or NPY KO BMNCs intravenously. (J) Survival of WT or NPY KO BMNCs transplanted WT mice (n = 15 per group). (K and L) Competitive repopulation assay showing the percentage of (K) CD45.2$^*$ cells in blood and the (L) percentage of LSK cells in BM 8 weeks after transplantation (n = 5 per group). (M) Percentage of donor CFU-C detected in the BM of $5 \times 10^6$ donor WT or NPY KO BMNCs transplanted WT mice at 24 hour after lethal irradiation (n = 4 per group). All error bars indicate s.e.m.]

“Response for manuscript 1”

3. Along the same line, the authors interpret that NPY produced by sympathetic nerve fibers has autocrine survival effects on these neurons and has also protective effects on endothelial cells. They assume that both of them would contribute to preserve HSC function. However the cause-effects relationships remain unclear, as whether these fibers are indeed the main source of NPY in the bone marrow. There is no demonstration of the claim in Figure 2 Legend title. Also, in the Discussion P.22 L.10: destruction of SNS fibers caused a decrease in the number of ECs, (also nestin$^+$ cells?)...some cause-effect relationships are withdrawn without much supportive evidence.

According to the referee’s comment, we have carefully reconsidered our manuscript. In our present study, we have focused on NPY produced by sympathetic nerve fibers regulating bone marrow function. As suggested by referee’s comment in question 2, we carefully investigated the possibility of bone marrow dysfunction by NPY deficiency in bone marrow cells. The results showed no significant difference in mouse survival, repopulating efficiency and the percentage of LSK cells in BM of WT or NPY KO BMNCs transplanted WT mice, as well as homing to BM (Figures. 2J-M in revised manuscript 1). These findings support that NPY produced by sympathetic nerve fibers preserves HSC function by regulating SNS fibers and endothelial cells.

In previous studies, 6OHDA treated mice, which induced destruction of SNS fibers, contained fewer CD31$^+$ ECs and nestin$^+$ cells after 5FU challenge, and the autonomic nervous system dysfunction affected the endothelial dysfunction (Nat Med. 2013, 19:695-703; Ann Vasc Dis. 2014, 7(2):109–119). Therefore, we thought that destruction of SNS fibers in BM of NPY$^-$ mice contributed to reduction of ECs. Moreover, we confirmed that NPY deficiency or Y1 receptor inhibition in ECs directly affected ECs apoptosis (Figure. 4F and Supplementary Figure. 2G in...
revised manuscript 1), suggesting NPY could mediate the survival of HSCs by regulating bone marrow SNS nerves and ECs.

As suggested by the referee’s comment, we have also carefully investigated whether destruction of SNS fibers caused a decrease in the number of ECs, as well as NPY expression. 6OHDA (100 mg/kg day 0 and 250 mg/kg day 2, i.p.) treated mice showed reduction of NPY expression and Th+ SNS fibers. The reduction of NPY expression co-localized with Th+ SNS fibers (Figures 3C-E in revised manuscript 1). Moreover, destruction of SNS fibers and low NPY expression in BM of 6OHDA treated mice caused a decrease in the number of ECs (Figure 3F in revised manuscript 1). Therefore, destruction of SNS fibers affected ECs survival as well as NPY expression. In revised manuscript 1 we have added these data as Figure 3C-F.

**Figure. 3C-F in revised manuscript 1. Destruction of SNS fibers caused a decrease in the number of ECs and NPY expression.** (C) Expression of NPY in BM of PBS or 6OHDA treated mice (n = 6 mice per group). (D) Quantification of Th+ fibers in the BM of PBS or 6OHDA treated mice (n = 4 mice per group). (E) Representative immunofluorescence images of BM showing NPY (green) merged with sympathetic neuron (Th, red). Scale bar, 40 mm. (F) Representative immunofluorescence BM images of CD31+ ECs and percentage of CD31+ ECs per femur in PBS or 6OHDA treated mice (Scale bar, 30 mm., n = 4 mice per group). *P < 0.05. All error bars indicate s.e.m.

4. Figure 2C shows a significant increase in apoptotic cells in the bone marrow of NPY KO mice. What are these cells? How do the authors reconcile increased apoptosis of bone marrow cells, increased quiescence of hematopoietic progenitors and yet no change in bone marrow cellularity? According to the referee’s comment, we have carefully reconsidered our manuscript. In Figure 1G in revised manuscript 1, we confirmed the percentage of apoptotic LSK cells using Annexin V antibody. The results showed no significant difference of LSK cells apoptosis in BM of NPY−/− mice compare to control mice. Also, Figure 1D and supplementary Figures 1A-D in revised manuscript 1 showed that the percentage of osteoblasts and the number of macrophages, T cells and B cells did not change in BM between two groups. These results indicated that increased apoptotic cells in BM of NPY−/− mice were not LSK cells, osteoblasts, macrophages, T cells and B cells.

In previous studies it was reported that Th+ SNS fibers and CD31+ endothelial cells (ECs) supported HSCs survival (Nat Med. 2013, 19:695-703). Next, we confirmed the number of Th+ SNS fibers and CD31+ ECs. The results showed reduction of bone marrow Th+ SNS fibers and CD31+ ECs in NPY−/− mice compared to control mice (Figure 3A and B in revised manuscript 1). To specifically confirm whether these cells are apoptotic cells in BM of NPY−/− mice, we performed TUNEL staining and western blot & real-time PCR for cell death pathway. Apoptotic Th+ cells or CD31+ ECs in vivo were increased in the BM of NPY−/− mice (Figure 4D in revised manuscript 1). NPY siRNA-exposed neurons or BM ECs in vitro showed increased TUNEL response and expression of p53 or pro-apoptotic gene (Figures 4F-H in revised manuscript 1). These findings indicated that the increased apoptotic cells in BM of NPY−/− mice are SNS fibers and CD31+ ECs.

NPY−/− mice showed that 20% more LSK cells were quiescent (in the G0 phase) compare to control mice. However, the S-G2-M phase did not change (Figure 1K in revised our manuscript 1). Of note, NPY deficiency did not alter the percentage of osteoblasts or the number of macrophages, B cells and T cells (Figure 1D and Supplementary Figures. 1A-D in revised manuscript 1). These results suggested that NPY deficiency did not affect HSCs differentiation in the bone marrow niche. Although apoptotic cells increased in BM of NPY−/− mice, its percentage was not sufficient to effect
of cellularity of total BM nuclear cells (Figure 4A in revised manuscript 1, Apoptotic cells in BM; CON : 0.09±0.02 %, NPY KO : 0.55±0.04 %).

“Response for manuscript 1”

5. The authors show very specific effects of NPY signaling in macrophages and osteoblasts on HSC survival/function and trafficking, respectively. How does Y1 receptor activation on macrophages (and not in osteoblasts) exert this specific function on HSCs?

Previous study reported that chemotherapy drugs such as cisplatin caused acute bone marrow injury, and impaired HSC function or bone marrow regeneration by reducing expression of Th⁺ fibers (Nat Med. 2013, 19:695-703; Cancer. 2001, 92:2419-2428; Exp Hematol. 2000, 28:1325-1333). We found that NPY-Y1 receptor regulation prevented HSC reduction by improving apoptosis of SNS fibers and niche cells in NPY⁻/⁻ mice (Figure 5 in revised manuscript 1), and cisplatin treated Lyz2-cre; Y1 fl/fl mice (Y1 receptor depleted mice in macrophages) did not show NPY-mediated protection of Th⁺ fibers and ECs, resulting in reduction of HSCs (Figure. 6 in revised manuscript 1). These results indicated that NPY promoted HSC survival through neuroprotection from cisplatin-induced bone marrow injury by stimulating Y1 receptor in macrophages.

To confirm the possibility of neuroprotection by NPY through Y1 receptor in macrophages, we have performed in vitro experiment of PC12 cells differentiation towards neurons after incubation with conditioned media (CM) derived from control macrophages or Y1 receptor deficient macrophages with or without NPY. Cells exposed to CM derived from NPY-treated Y1 receptor deficient macrophages did not show neural differentiation capacity (Supplementary Figure. 3A in revised manuscript 1), suggesting neuroprotection by NPY is mediated by factors secreted from Y1 receptor-stimulated macrophages.

According to the referee’s comment, we have carefully investigated and confirmed which factor released from macrophages induces neuroprotection by NPY-mediated Y1 receptor stimulation. We screened and compared the CM of control or Y1 receptor deficient macrophages with and without NPY for 50 different secreted cytokines using an antibody-based mouse cytokine array (Supplementary Figure. 3B in revised manuscript 1). The CM of control macrophages treated with NPY revealed stronger signals in 4 array spots in comparison to the CM of control macrophages alone (Supplementary Figure. 3C in revised manuscript 1). We also confirmed the expression levels of these factors in each cells, and found up-regulated TGF-b expression in control macrophages treated with NPY. However, Y1 receptor deficient macrophages treated with NPY did not increase TGF-b expression both CM and cells (Supplementary Figures. 3D and E in revised manuscript 1).

TGF-b holds key roles in regulation of neuronal survival and orchestration of repair processes in the nervous system, and is produced through the PI3K/Akt/mTOR/eIL4E signaling pathway (Journal of Physiology-Paris. 2002, 96:25-50; Journal of Immunology. 2008, 181:3575-3585; Neurobiology of Disease. 2010, 38:395–404). In another study it was reported that NPY promoted TGF-b production by activating the PI3K pathway via Y1 receptor (Neuroscience bulletin. 2008, 24:155-159).

Based on these papers and our data, we confirmed the role of the PI3K pathway in control macrophages or Y1 receptor deficient macrophages with and without NPY. The results indicated that PI3K levels increase in control macrophages with NPY compared to control macrophages alone. Downstream signaling also was activated, but not in Y1 receptor deficient macrophages treated with NPY (Supplementary Figure. 3F in revised manuscript 1). These findings suggested that TGF-b released by NPY-mediated Y1 receptor stimulation in macrophages promoted neuroprotection from cisplatin-induced injury. However, further studies are needed to investigate a more specific role of TGF-b secreted from macrophages in the bone marrow niche. To better convey and clarify the message of our study, we have added these data as Supplementary Figure. 3B-F in revised manuscript 1.
Supplementary Figure. 3B-F in revised manuscript 1. NPY induces TGF-b production though Y1 receptor-mediated PI3K pathway in macrophages. (B) The names and locations of each cytokine/chemokine custom spot for this set of experiments are listed. (C) The boxed areas indicate up-regulated proteins in the CM derived from control macrophages with NPY. (D) Films were scanned and analyzed using the Bio-Rad analysis software. The optical intensity of the cytokine spots of interest (boxed) was quantified. The average optical intensity for each pair of listed cytokine spots is shown. (E) mRNA levels of cytokines of interest in control or Y1 receptor deficient macrophages with or without NPY (n = 3). (F) Western blot analysis and quantification for PI3K, p-Akt, p-mTOR and p-eIF4E in each group (n = 3). *P < 0.05. All error bars indicate s.e.m.

“Response for manuscript 1”

6. How do the authors explain the apparently contradictory results in NPY KO mice that show increased SDF-1, Angpt1 and Kitl BM mRNA levels but no difference in HSPC homing?

According to the referee’s comment, we have carefully reconsidered our manuscript. In our present study, we performed a HSC homing assay to investigate whether dysfunction of bone marrow regeneration in NPY−/− mice was due to impairment of HSC engraftment in the BM after transplantation. Although BM of NPY−/− mice exhibited up-regulated levels of SDF-1, Angpt1 and Kitl compared to WT mice in normal conditions, these mice were irradiated lethally for BM transplantation. We hypothesized that lethal irradiation was able to destroy the original BM cells expressing these HSC maintenance factors. To confirm this possibility, BM cells from WT or NPY−/− mice was extracted to check expression of HSC maintenance factors after irradiation (two split doses of 5 Gy). The results indicated down-regulated expression of these factors after irradiation compared to non-irradiated mice (Additional Figure. 2). Therefore, we suggest that increased HSC maintenance factors in BM of NPY−/− mice did not affect HSPC homing due to reduction of these factors by irradiation similar to WT mice’s.

Additional Figure. 2. Expression of HSC maintenance factors in BM of WT or NPY−/− mice received irradiation or not. (n = 4 per group). *P < 0.05. All error bars indicate s.e.m.

“Response for manuscript 1 or 2”
7. The NPY dose injected in mice seems higher than the physiological range, questioning whether some responses would be expected to occur normally in vivo.

We guess that some responses are induced by NPY levels of physiological range. Further studies are needed to confirm the responses by NPY normally in vivo. NPY is known as a neurotransmitter that is often released at the sympathetic nerve in central or peripheral nervous system, and as an orexigenic peptide inducing food intake normally. However, NPY is upregulated by acute or chronic stress, and contributes to reduction of anxiety and pain under highly physiological range (Neuropeptides. 2004, 38:201-211; Peptides. 2007, 28:435-440. Therefore, we expect that the responses in our study would occur under highly physiological range of NPY.

Importantly, in present our study high does treatment of NPY improved chemotherapy induced BM injury and bone loss of ovariectomized mice under pathological conditions. These findings provide that NPY has potential clinical utility as neuroprotective agent for chemotherapy, and also as a therapeutic agent for osteoporosis. Therefore, we thought that the modulation of endogenous bone marrow HSC niche by NPY could be a more effective therapeutic approach than direct transplantation using bone marrow or stem cells. These findings may provide new insights to the field of stem cell research or cell therapy for clinical application.

“Response for manuscript 1”

8. How do NPY receptor levels compare between osteoblasts, endothelial cells and nestin+ cells? Can the different response of these cells to NPY be explained by different of the receptor?

According to the referee’s comment, we evaluated the expression of the Y1 receptor in macrophages, osteoblasts, endothelial cells and nestin+ cells. Macrophages and osteoblasts, endothelial cells and nestin+ cells were sorted from BM of WT or Nestin-GFP mice through flow cytometry. Expression of the Y1 receptor in macrophages and osteoblasts tended to increase compared to endothelial cells and nestin+ cells (Figure. 6A in revised manuscript 1). Therefore, we focused on Y1 receptor in these cells to reveal the mechanism of NPY-mediated BM recovery in chemotherapy induced BM injury. In the revised manuscript 1, we have added this data as Figure. 6A. In our present study, NPY promoted HSC survival through neuroprotection from cisplatin-induced bone marrow injury by stimulating Y1 receptor in macrophages. Moreover, we found that TGF-β secreted from macrophages by NPY-mediated Y1 receptor stimulation induced neuroprotection from cisplatin-induced injury (Supplementary Figures. 3B-F in revised manuscript 1). Y1 receptor in osteoblasts mediated HSC mobilization by regulating HSC maintenance factors. We also confirmed increased osteoblasts and MSCs (origin of osteoblast) in BM of NPY treated mice (Supplementary Figure. 1A). These findings contributed to improvement of bone loss in ovariectomized mice. Previous studies reported that NPY induced proliferation and differentiation of MSCs, supporting these results (Am J Physiol Heart Cir Physiol. 2010, 298:H275-286).

Endothelial cells showed reduction in BM of NPY−/− mice, and this was protected by NPY or Y1 agonist treatment (Figure. 5 in revised manuscript 1). These results were also supported by previous studies showing that NPY promoted endothelial cell growth and proliferation by regulating Y1 receptors (Peptides. 1993, 14(2):263-8; Canadian Journal of Physiology and Pharmacology. 2003, 81(2):177-185; Canadian Journal of Physiology and Pharmacology. 2008, 86(7):438-48). However, further studies are needed to investigate more specific response of these cells to NPY through the Y1 receptor.

Figure. 6A in revised manuscript 1. Expression of Y1 receptor in macrophages, osteoblasts, endothelial cells and nestin+ cells. (n = 3 of cultured macrophage, and three experiments in which populations (osteoblasts, endothelial cells and nestin+ cells) were sorted from BM of ten mice each.). All error bars indicate s.e.m.

“Response for manuscript 1”
9. Figure 1 Legend title seems wrong.
According to the referee’s comment, we agree and found that the legend could cause misunderstandings. We have revised the Figure 1 legend title in manuscript 1 (p.30 of the revised manuscript 1).

Referee #2:
The authors investigate the role of NPY on hematopoietic dynamics with extensive analysis of gain and loss of function models. They show that NPY leads to HSC egress from the bone marrow and, conversely, deletion of the NPY receptor is protective. These effects are mediated by the of known HSC niche molecular components, such as SDF1, angiopoietin 1 and Kit ligand. Overall the data are extensive and the work is novel. The biggest issue for the reader is to find the right way through the mass of data, with the explanation of the experiments being not always as clear as it could be (some examples below). Rewriting completely the manuscript with a more clear focus and more succinct figures will greatly increase the readability and will communicate a more clear message. There are however some considerable concerns that the authors should address/rectify.

We would like to thank the referee for the comments concerning our manuscript. In accordance with these comments, we have separated the data into two manuscripts to focus on a more specific message and readability. Manuscript 1 demonstrates HSC impairment due to NPY deficiency and the potential therapeutic application of NPY in chemotherapy-induced bone marrow nerve injury.

“Response for manuscript 1”
1. One considerable oddity is the low level of engraftment observed in all transplantation experiments described (Figure 4L-N, 10^6 BMNC should give rise to 95% of chimerism, not 1.5%; Figure 5 L-N, a 1:1 competitive transplant should lead to 50% chimerism, not 10%).

According to the referee’s comment, we have carefully reconsidered our manuscript. In original Figure 4L (Figure. 7A in revised manuscript 1) we performed bone marrow transplantation to evaluate whether NPY treatment prevents cisplatin-induced mice death and impairment of BM after transplantation. 1x10^6 BMNCs were transplanted into cisplatin treated mice with or without NPY treatment after lethal irradiation. NPY treatment enhanced mouse survival and rescued cisplatin-induced bone marrow dysfunction after transplantation (Figures. 7B-C in revised manuscript 1). The original Figure 4N (Figure. 7C in revised manuscript 1) indicated that the count of BMNCs was 1.5x10^6 cells in BM of cisplatin treated mice with or without NPY treatment after transplantation, not 1.5% of chimerism. This count of BMNCs was similar to the count in previous study (Nat Med. 2013, 19:695-703). To confirm successful chimerism, we have re-performed 1:1 competitive transplants as shown original Figure 5N). The results showed 44.15% of chimerism in blood of recipient mice (Additional Figure. 3).

Additional Figure. 3. The chimerism of 1:1 competitive transplantation. 2x10^6 CD45.2+ bone marrow cells from PBS or NPY treated mice were transplanted into lethally irradiated CD45.1 recipients together with an equal number of competing CD45.1+ bone marrow cells. After 8 weeks, the percentage of CD45.2+ donor derived cells were measured by flow cytometry in PB and BM (n = 4 mice per group). *P < 0.05. All error bars indicate s.e.m.
“Response for manuscript 1”

2. It is also not clear how Lyz2-cre would target only perivascular macrophages but not, for example, macrophages localized near osteoblasts. Either this point is proved or the description of the mouse strain and the conclusions made should be changed.

According to the referee’s comment, Lyz2-cre recombined in the myeloid cell lineage targets not only perivascular macrophages but also macrophages localized in other bone marrow areas. Although these mice target all macrophage subtypes, we could not find suitable perivascular macrophage specific-cre mice. As suggested by this referee’s comment, we have revised the sentence in the result section for clarification (p.12 of the revised manuscript 1).

“Response for manuscript 1”

3. In Figure 10 it seems the colonies are 95% of host origin, hence showing no significant difference. Colony assays following better engraftment would be more informative.

According to the referee’s comment, we have carefully reconsidered our manuscript. In the present study, we confirmed impairment of bone marrow regeneration in NPY−/− mice through bone marrow transplantation (Figures. 2A-C in revised manuscript 1). Engraftment of transplanted bone marrow cells requires homing to bone marrow and migration to the appropriate niche. We therefore performed a homing assay in transplanted WT and NPY−/− mice as shown in original Figure 1M-O (Figures. 2D-F in revised manuscript 1).

The original Figure 1N (Figure. 2E in revised manuscript 1) showed that the percentage of donor CFU-C detected in the BM of WT or NPY−/− mice at 24 h after lethal irradiation and injection of 5×10⁶ donor WT BMNCs, and original Figure 1O (Figure. 2E in revised manuscript 1) indicated that the percentage of CD45.1+ cells in peripheral blood (PB) of CD45.2+ recipient mice 16 weeks after competitive transplant. The results showed no significant difference between two groups, suggesting that impaired bone marrow regeneration in NPY−/− mice is independent of homing. We have referred to previous study for these experiments, and confirmed that the percentage of donor CFU-C detected in the BM of WT mice and CD45.1+ cells in PB of CD45.2+ recipient mice were similar to the percentages of reference (Nat Med. 2013, 19:695-703; Additional Figure. 4).

Additional Figure. 4. HSPC homing efficiency. (a) Experimental design to determine the efficiency of HSC homing to the BM. (b) Percentage of donor CFU-C detected in the BM 24 h after lethal irradiation (1200 rads) and injection of 5×10⁶. (c) Percentage of CD45.1+ cells in peripheral blood of CD45.2+ recipient mice 16 weeks after competitive transplant as depicted in a.

“Response for manuscript 1”

5. Figure 2C: it is not clear what BM cells were assessed for apoptosis.

According to the referee’s comment, we have carefully reconsidered our manuscript. In Figure 1G in revised manuscript 1, we analyzed the percentage of apoptotic LSK cells using Annexin V antibody to confirm the mechanism leading to LSK cells and LT-HSCs reduction in BM of NPY−/−
mice. The results showed no significant difference of LSK cells apoptosis in BM of NPY−/− mice compared to control mice. Also Figure 1D and supplementary Figures 1A-D in revised manuscript 1 show that the percentage of osteoblasts and the number of macrophages, T cells and B cells did not change in BM between the two groups. These results indicated that increased apoptotic cells in BM of NPY−/− mice were not LSK cells, osteoblasts, macrophages, T cells and B cells.

A previous study reported that Th⁺ SNS fibers and CD31⁺ endothelial cells (ECs) supported HSCs survival (Nat Med. 2013, 19:695-703). Next, we confirmed the number of Th⁺ SNS fibers and CD31⁺ ECs. The results showed reduction of bone marrow Th⁺ SNS fibers and CD31⁺ ECs in NPY−/− mice compared to control mice (Figures. 3A and B in revised manuscript 1). To specifically confirm whether these cells are apoptotic cells in BM of NPY−/− mice, we performed TUNEL staining and western blot & real-time PCR for the cell death pathway. Apoptotic Th⁺ cells or CD31⁺ ECs in vivo were increased in the BM of NPY−/− mice (Figure. 4D in revised manuscript 1). NPY siRNA-exposed neurons or BM ECs in vitro showed increased TUNEL response and expression of p53 or pro-apoptotic genes (Figures. 4F-H in revised manuscript 1). These findings indicated that increased apoptotic cells in BM of NPY−/− mice are SNS fibers and CD31⁺ ECs.

“Response for manuscript 1”

6. Figure 4E: NPY treatment recover chemotherapy-induced HSC impairment only in Col-cre mice, not in all strains.

According to the referee’s comment, we have carefully reconsidered our manuscript. In this study, we found that bone marrow impairment in NPY−/− mice was recovered by NPY or Y1 agonist treatment (Figure. 5 in revised manuscript 1). Based on these findings, we predicted that NPY might protect against bone marrow dysfunction from chemotherapy-induced injury through the Y1 receptor. To investigate this hypothesis, we first depleted the Y1 receptor in macrophages and osteoblasts, conditionally using a Lyz2-cre, co11a1-cre and Y1fl/fl breeding system to evaluate which niche cell mediated protection from chemotherapy-induced injury. Chemotherapy-induced HSC impairments were recovered by NPY treatment in control and co11a1-cre; Y1fl/fl mice. However, Lyz2-cre; Y1fl/fl mice did not show NPY-mediated protection of HSC impairments (Figure. 6F in revised manuscript 1). These results suggested that NPY promoted neuroprotection and bone marrow dysfunction from cisplatin-induced injury through Y1 receptors in macrophages. To clarify this finding, we have revised the result section.

“Response for manuscript 1”

7. Figure 2G-J: in vitro cultures of cell lines are an experimental model that is very far from in vivo neurons. This is an example of data that does not majorly strengthen the manuscript and could be moved to a supplement.

We agree. As suggested by this referee’s comment, PC12 cell lines used in original Figures 2G-J (Figures. 4E-H in revised manuscript 1) are far from in vivo neurons. We found that the BM of NPY−/− mice contained fewer Th⁺ nerves compared to WT mice (Figure. 3A in revised manuscript 1), and we wanted to know how NPY deficiency affects the survival of bone marrow Th⁺ nerves. However, it is very difficult to culture neuron from bone marrow.

The PC12 cell line may be differentiated to neurons with neurotrophic factors (ex, nerve growth factor). In previous reports, this cell line was used to confirm which agent protects bone marrow nerves from chemotherapy-induced nerve injury in vitro (Nat Med. 2013, 19:695-703.). Therefore, we used the PC12 cell line based on this concept, and found that NPY deficiency in neurons differentiated from PC12 cells caused p53-dependent apoptosis.
Referee #3:

In this interesting study the authors show the involvement of NPY and its receptor in HSC repopulation, maintenance and mobilization. They look at potential effects of NPY on macrophages, osteoclasts, nerve and endothelial cells. By using different transgenic mouse models they show that NPY deficiency causes BM impairments of HSC survival and mobilization, as well as integrity of SNS nerve fibers. Pharmacological or endogenous NPY increase leads to HSC regeneration and mobilization through the Y1 receptor. In addition, NPY treatment prevents cisplatin-induced deficits in BM function and ovariectomy-induced bone loss in mice through the Y1 receptor. Although, the authors use a lot of assays, murine models and tools as well as show everything in several ways, no novel mechanistic insights are shown in the manuscript. The only suggested regulator is p53 which is shown only in a correlative way. Dependency on p53 is not proved by the data. In addition, the manuscript is too loaded with data and difficult to follow. Therefore, I suggest that the authors should choose and focus on a specific message and re-edit the manuscript accordingly. For example, the manuscript may be divided into two different papers: one dealing with mobilization and the other with HSC maintenance and stress-induced response. There are too many results and the authors are encouraged to consider splitting the MS into 2 studies, 1 focused on stem cell maintenance and mobilization and the other on bone and bone marrow integrity and how to overcome osteoporosis.

We would like to thank the referee for the comments concerning our manuscript. In accordance with these comments, we have separated the data into two manuscripts to focus on a more specific message and readability. Manuscript 1 demonstrates HSC impairment due to NPY deficiency and the potential therapeutic application of NPY in chemotherapy-induced bone marrow nerve injury.

“Response for manuscript 1”

1. In their results the authors observed that NPY −/− mice exhibit reduced numbers of HSPC in the BM and in the PB, in addition to reduced numbers of niche elements such as Sca-1+ MSPC. The conclusion made by the authors that the reduced numbers of HSPC in the PB is due to reduced HSPC egress might be wrong as reduced numbers of HSPC in the PB may be derived from their reduced numbers in the BM. Assays to examine BM HSPC motility and migration capacity should be performed to enlighten this aspect and help to make a final conclusion.

According to the referee’s comment, we found reduction in the numbers of HSPC in the PB and BM of NPY−/− mice (Figure. 1C and E in revised manuscript 1). To more carefully confirm whether reduced numbers of HSPC in the PB were derived from their reduced numbers in the BM, we performed migration assays. Five-thousand LSK cells sorted from BM of WT or NPY−/− mice were placed on the top chamber of a 24-well transwell plate (Corning). Recombinant SDF-1α (100 ng/ml, R&D System) was placed in the bottom wells, and the plate was incubated at 37°C, 5% CO₂ for 4 hours. Cells that had migrated to the lower chamber were visualized and enumerated using Olympus IX71 microscope. The results showed no significant difference between migrated LSK cells of WT and NPY−/− mice (Figure. 1F in revised manuscript 1). Therefore, NPY deficient HSPCs had normal motility and migration capacity, suggesting that reduction of HSPC in the PB in NPY−/− mice were derived from their reduced numbers in the BM. In revised manuscript 1 we have added these results to better convey our message as Figure. 1F.
"Response for manuscript 1"

3. The authors provide evidence that NPY deficiency results with enhanced apoptosis of BM cells and conclude that apoptosis of niche elements are leading to reduced HSPC numbers. However this may be derived from total apoptosis effects which are general for all BM cells including HSPC and is not only dependent on niche function and availability. How can the authors conclude that the reduced HSPC numbers is not due to cell-autonomous effect in which NPY deficiency leads to increased apoptosis among HSPC? Please test direct effects of NPY on enriched HSPC in vitro.

According to the referee’s comment, we have carefully reconsidered our manuscript. In Figure 1G in revised manuscript 1, we confirmed the percentage of apoptotic LSK cells in BM using Annexin V antibody. The results showed no significant difference of LSK cells apoptosis in BM of NPY−/− mice compare to control mice. Also, Figure 1D and supplementary Figures 1A-D in revised manuscript 1 showed that the percentage of osteoblasts and the number of macrophages, T cells and B cells did not change in BM between the two groups. These results indicated that increased apoptotic cells in BM of NPY−/− mice were not LSK cells, osteoblasts, macrophages, T cells and B cells.

Moreover, we found reduction of bone marrow Th+ SNS fibers and CD31+ ECs in NPY−/− mice compared to control mice (Figures. 3A and B in revised manuscript 1). To specifically confirm whether these cells are apoptotic cells in BM of NPY−/− mice, we performed TUNEL staining and western blot & real-time PCR for the cell death pathway. Apoptotic Th+ cells or CD31+ ECs in vivo were increased in the BM of NPY−/− mice (Figure. 4D in revised manuscript 1). NPY siRNA-exposed neurons or BM ECs in vitro showed increased TUNEL+ response and expression of p53 or pro-apoptotic genes (Figures. 4F-H in revised manuscript 1). These findings indicated that increased apoptotic cells in BM of NPY−/− mice are SNS fibers and CD31+ ECs.

As suggested by this referee’s comment, we more specifically investigated whether reduced HSPC numbers in NPY−/− mice was a cell-autonomous effect in vitro. LSK cells sorted from BM of WT mice were treated with control or NPY siRNA for 48 hours, and we checked reduction of NPY expression (Figure. 1H in revised manuscript 1). To confirm apoptotic LSK cells, Annexin V was stained in control or NPY siRNA treated LSK cells and analyzed through flow cytometry. The results showed no significant difference in apoptosis, and expression of pro-apoptotic genes also was not changed between the two groups (Figure. 1I and J in revised manuscript 1). Therefore, NPY deficiency did not affect cell-autonomous apoptosis in HSCs. In revised manuscript 1, we have added these results to better convey our message as Figure. 1H-J.
Figure. 1H-J in revised our manuscript 1. NPY deficiency did not affect cell-autonomous apoptosis in HSCs. (H) Expression levels of NPY in LSK cells exposed to control or NPY siRNA (n = 3). (I) Percentage of apoptotic cells in control or NPY siRNA treated LSK cells (n = 3 mice group). (J) Quantitative real-time PCR analysis for pro- or anti-apoptotic gene expression in each group (n = 3). *P < 0.05. All error bars indicate s.e.m.

4. The authors introduce macrophages as part of the affected niche in their study. Yet, the markers defining macrophages that are used by the authors actually label a broad myeloid population. Recently a very small population of alphaSMA expressing macrophages was shown to maintain BM HSPC under steady state and during stress induced conditions such as myeloablative irradiation treatment which requires BM recovery (Ludin et al., Nature Immunology 2013 (please cite)). What is the status of these cells under NPY deficiency of when total myeloid Lyz2-Cre system is activated to delete NPY receptor? What is their contributions to HSPC egress and mobilization as HSPC anchoring cells in the BM? What is the role of this population in nerve fiber recovery?

“Response for manuscript 1”

4-1. What is the status of these cells under NPY deficiency of when total myeloid Lyz2-Cre system is activated to delete NPY receptor?

According to the previous study recommended by the referee (Nat Immunology. 2012, 13(11):1072–82), we carefully investigated the percentage of aSMA expressing macrophages in BM of WT, NPY−/− or Lyz2-cre;Y1fl/fl mice. The results showed no difference in the percentage of aSMA+MAC1+ macrophages in BM of each group (Supplementary Figure. 1C in the revised manuscript 1 and Additional Figure 5). These finding indicated that HSC maintained by aSMA+MAC1+ macrophages did not affect deficiency of NPY or Y1 receptor. We have cited and commented about the previous study, and added the data of WT and NPY−/− mice as Supplementary Figure. 1C in the revised manuscript 1.

Supplementary Figure. 1C in the revised manuscript 1. aSMA+MAC1+ macrophages did not affect deficiency of NPY. Percentage of and aSMA+MAC1+ macrophages in BM of (A) WT or NPY−/− (n = 4 per group). All error bars indicate s.e.m.
“Response for manuscript 1”

4-3. What is the role of this population in nerve fiber recovery?

To investigate the role of αSMA expressing macrophages in nerve fiber recovery, in vitro experiment of PC12 cells differentiation towards neurons could be performed after incubation with conditioned media (CM) derived from αSMA expressing macrophages with or without NPY. However, it’s difficult to sort these cell population. Because BM contained a very small population of these cells (0.1% of total bone marrow cells in previous study; Nat Immunology. 2012, 13(11):1072-82). To overcome this difficulty, the previous study used αSMA-RFP transgenic mice in various experiments. Although we did not have these mice, we thought that αSMA+ macrophages might affect SNS fiber recovery in BM of chemotherapy treated mice. As suggested previous study, these cells required to protect the pool of HSPCs from exhaustion on the steady state, and maintain HSPCs with their adjacent stromal and myeloid cells near blood vessels. Therefore, αSMA+ macrophages also might mediate improvement of bone marrow dysfunction from chemotherapy. However, further studies are needed to investigate more specific response of these cells in nerve fiber recovery.

“Response for manuscript 1”

7. The effect of NPY in macrophages that enables to escape BM failure following chemotherapy is shown to be mediated by NPY1R. Which signaling pathway does this involve?

According to the referee’s comment, we have carefully reconsidered our manuscript. We confirmed that NPY/Y1 receptor regulation affected HSC survival by improving apoptosis of SNS fibers and niche cells in NPY−/− mice (Figure. 5 in revised manuscript 1), and chemotherapy-induced HSC impairments were recovered by NPY treatment in cisplatin treated WT or collagen-cre; Y1fl/fl mice (Y1 receptor depleted mice in osteoblasts). However, cisplatin treated Lyz2-cre; Y1fl/fl mice (Y1 receptor depleted mice in macrophages) did not show NPY-mediated protection of Th+ fibers and ECs, resulting in reduction of HSCs (Figure. 6 in revised manuscript 1). These results indicated that NPY promoted HSC survival through neuroprotection from cisplatin-induced BM failure by stimulating Y1 receptors in macrophages.

To confirm the possibility of neuroprotection by NPY through Y1 receptor in macrophages, we have performed in vitro experiments evaluating PC12 cell differentiation towards neurons after incubation with conditioned media (CM) derived from control macrophages or Y1 receptor deficient macrophages with or without NPY treatment. Cells exposed to CM derived from NPY-treated Y1 receptor deficient macrophages did not show neural differentiation capacity (Supplementary Figure. 3A in revised manuscript 1), suggesting neuroprotection by NPY is mediated by factors secreted from Y1 receptor-stimulated macrophages.

Additional Figure. 5. αSMA+MAC1+ macrophages did not affect deficiency of NPY or Y1 receptors. Percentage of and αSMA+MAC1− macrophages in BM of +/+ or Lyz2-cre; Y1fl/fl mice. (n = 4 per group). All error bars indicate s.e.m.
According to the referee’s comment, we have carefully investigated and confirmed which factor released from macrophages induces neuroprotection by NPY-mediated Y1 receptor stimulation. We screened and compared the CM of control or Y1 receptor deficient macrophages with and without NPY for 50 different secreted cytokines using an antibody-based mouse cytokine array (Supplementary Figure 3B in revised manuscript 1). The CM of control macrophages treated with NPY revealed stronger signals in 4 array spots in comparison to the CM of control macrophages alone (Supplementary Figure. 3C in revised manuscript 1). We also confirmed the expression levels of these factors in each cells, and found up-regulated TGF-b expression in control macrophages treated with NPY. However, Y1 receptor deficient macrophages treated with NPY did not increase TGF-b expression in both CM and cells (Supplementary Figures. 3D and E in revised manuscript 1).


Based on these papers and our data, we confirmed the role of the PI3K pathway in control macrophages or Y1 receptor deficient macrophages with and without NPY. The results indicated that PI3K levels increased in control macrophages treated with NPY treatment compared to control macrophages alone. Downstream signaling also was activated, but not in Y1 receptor deficient macrophages treated with NPY (Supplementary Figure. 3F in revised manuscript 1). These findings suggested that TGF-b released by NPY-mediated Y1 receptor stimulation in macrophages promoted neuroprotection from cisplatin-induced injury. However, further studies are needed to investigate more specifically investigate the role of TGF-b secreted from macrophages in maintenance of the bone marrow niche. To better convey and clarify the message of our study, we have added these data as Supplementary Figure. 3B-F in revised manuscript 1.

**Supplementary Figure. 3B-F in revised manuscript 1.** NPY induces TGF-b production though Y1 receptor-mediated PI3K pathway in macrophages. (B) The names and locations of each cytokine/chemokine custom spot for this set of experiments are listed. (C) The boxed areas indicate up-regulated proteins in the CM derived from control macrophages with NPY. (D) Films were scanned and analyzed using the BioRad analysis software. The optical intensity of the cytokine spots of interest (boxed) was quantified. The average optical intensity for each pair of listed cytokine spots is shown. (E) mRNA levels of cytokines of interest in control or Y1 receptor deficient macrophages with or without NPY (n = 3). (F) Western blot analysis and quantification for PI3K, p-Akt, p-mTOR and p-eIF4E in each group (n = 3). *P < 0.05. All error bars indicate s.e.m.
“Response for manuscript 1”

10. More data should be added on the mechanism or the factors from the macrophages that mediate neural protection in BM niche.

Previous studies reported that chemotherapy drugs such as cisplatin cause acute bone marrow injury and impair HSC function or bone marrow regeneration by reducing expression of Th⁺ fibers (Nat Med. 2013, 19:695-703; Cancer. 2001, 92:2419-2428; Exp Hematol. 2000, 28:1325-1333).

We found that NPY/Y1 receptor regulation affected HSC survival by improving apoptosis of SNS fibers and niche cells in NPY⁻/⁻ mice (Figure. 5 in revised manuscript 1), and cisplatin treated Lyz2-cre; Y1[fl/fl] mice (Y1 receptor depleted mice in macrophage) did not show NPY-mediated protection of Th⁺ fibers and ECs, resulting in reduction of HSCs (Figure. 6 in revised manuscript 1). These results indicated that NPY promoted HSC survival through neuroprotection from cisplatin-induced bone marrow injury by stimulating Y1 receptors in macrophages.

To confirm the possibility of neuroprotection by NPY through Y1 receptor in macrophages, we have performed in vitro experiments evaluating PC12 cell differentiation towards neurons after incubation with conditioned media (CM) derived from control macrophages or Y1 receptor deficient macrophages with or without NPY treatment. Cells exposed to CM derived from NPY-treated Y1 receptor deficient macrophages did not show neural differentiation capacity (Supplementary Figure. 3A in revised manuscript 1), suggesting neuroprotection by NPY is mediated by factors secreted from Y1 receptor-stimulated macrophages.

According to the referee’s comment, we have carefully investigated and confirmed which factor released from macrophages induces neuroprotection by NPY-mediated Y1 receptor stimulation. We screened and compared the CM of control or Y1 receptor deficient macrophages with and without NPY for 50 different secreted cytokines using an antibody-based mouse cytokine array (Supplementary Figure. 3B in revised manuscript 1). The CM of control macrophages treated with NPY revealed stronger signals in 4 array spots in comparison to the CM of control macrophages alone (Supplementary Figure. 3C in revised manuscript 1). We also confirmed the expression levels of these factors in each cells, and found up-regulated TGF-β expression in control macrophages treated with NPY. However, Y1 receptor deficient macrophages treated with NPY did not increase TGF-β expression both CM and cells (Supplementary Figures. 3D and E in revised manuscript 1).


Based on these papers and our data, we confirmed the role of the PI3K pathway in control macrophages or Y1 receptor deficient macrophages with and without NPY treatment. The results indicated that PI3K levels increased in control macrophages with NPY compared to control macrophages alone. Downstream signaling also was activated, but not in Y1 receptor deficient macrophages treated with NPY (Supplementary Figure. 3F in revised manuscript 1). These findings suggested that TGF-β released by NPY-mediated Y1 receptor stimulation in macrophages promoted neuroprotection from cisplatin-induced injury. However, further studies are needed to investigate a more specifically the role of TGF-β secreted from macrophages in the bone marrow niche. To better convey and clarify the message of our study, we have added these data as Supplementary Figure. 3B-F in revised manuscript 1.
Supplementary Figure. 3B-F in revised manuscript 1. NPY induces TGF-b production though Y1 receptor-mediated PI3K pathway in macrophages. (B) The names and locations of each cytokine/chemokine custom spot for this set of experiments are listed. (C) The boxed areas indicate up-regulated proteins in the CM derived from control macrophages with NPY. (D) Films were scanned and analyzed using the Bio-Rad analysis software. The optical intensity of the cytokine spots of interest (boxed) was quantified. The average optical intensity for each pair of listed cytokine spots is shown. (E) mRNA levels of cytokines of interest in control or Y1 receptor deficient macrophages with or without NPY (n = 3). (F) Western blot analysis and quantification for PI3K, p-Akt, p-mTOR and p-eIF4E in each group (n = 3). *P < 0.05. All error bars indicate s.e.m.

Minor comments

1. The authors should avoid statements like "for the first time" and also should be more modest about their conclusions.

According to the referee’s comment, we have reconsidered and revised our manuscript.

2. The authors over-claim that NPY might be a treatment for osteoporosis. There were no human subjects in this work with osteoporosis that were given NPY.

According to the referee’s comment, we have reconsidered and revised our manuscript.

3. In figure 5.D, the authors can add "NPY" to the graph of Osteoblast differentiated from BM-MSC, to make it clearer.

According to the referee’s comment, we have reconsidered and rearranged our manuscript. We have added "NPY" to the graph in Supplementary Figure 3B.

4. The figures are very crowded with a lot of data and many graphs therefore, it is very difficult to
follow them. More clarity and less fig. are encouraged, in line with our suggestion to split the data into 2 MS.

According to the referee’s comment, we have separated the data into two manuscripts to focus on a more specific message and readability. *Manuscript 1* demonstrates HSC impairment due to NPY deficiency and the potential therapeutic application of NPY in chemotherapy-induced bone marrow nerve injury.

2nd Editorial Decision 02 March 2015

Thank you very much for your revision, respective splitting the original submission into two separate studies. These have been independently assessed from two of the original referees.

As you will recognize from the enclosed comments, there are a few remaining items that I kindly ask you to attend.

Specifically:

- please refer in the title, abstract and the entire paper (90174R) to the role of NPY on the MICROENVIRONMENT/bone marrow stroma, rather than the 'niche' to increase the distinction from the related study (91130).
- please address the major and minor points as raised from ref#3 in the final round of peer-review before submitting an ultimate version of this paper for ultimate acceptance/publication in The EMBO Journal.

Further:

- The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels/blots, with the aim to make primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans for KEY data of published work. We would be grateful for one PDF-file per figure with this information.

- Please include a conflict of interest statement into the text file.

- Please also provide a minimal 2 up to 4 'bullet point' synopsis, as to highlight the major novelty/advance provided by your study.

- If you were to have an integrating figure as to visualize this in the format of 550x150 (400max) pixel, this would facilitate featuring your study on our homepage upon formal publication.

I am very much looking forward to receive your final amendments as well as the specified items together with your final revisions.

Please allow me already at this stage to congratulate you to a very insightful study.

REFEREE REPORTS:

Referee #3:

Neuropeptide Y regulates the hematopoietic stem cell niche and prevents nerve injury in the bone marrow

In this study the authors show how stem cells in the bone marrow are maintained by NYP via indirect regulation of the stromal microenvironment. By using different transgenic mouse models they show that NPY deficiency causes bone marrow impairments of HSC survival, as well as
integrity of SNS nerve fibers and ECs. Pharmacological or endogenous NPY increase leads to HSC regeneration through the Y1 receptor. The fact that the authors divided their original paper into two papers improved very much the quality and focus of each paper. In addition, the authors invested a lot of hard work and managed to answer most of reviewer's comments. The molecular mechanistic aspects although improved, still need to be a bit fine tuned. Therefore I would like the authors to refer to the following comments.

Major comments
1. In a previous publication by the group of Nakauchi H (Cell, 2011, Yamazaki S et al) it was shown that non-myelinated schwann cells maintain stem cells in the BM niche by activating TGFb. Please cite this work as well as test if Schwann cells in WT mice express NPY receptor, which in turn will induce TGFb secretion.
2. The regulation of ECM embedded TGFb by osteoclasts and its role in Sca-1 bone forming progenitor cells was already shown. Please cite this paper and discuss it (X. Wu and Xu Cao in Cell Stem Cells 2010).

Minor comments
1. In supplementary fig. 3, the authors claim that the TGFb downstream pathway is activated. Please write what is this pathway.
2. In the supplementary part quantitative PCR was done from isolated RNA from B...? Which cells the authors refer to?

Referee#3:
Neuropeptide Y regulates the hematopoietic stem cell niche and prevents nerve injury in the bone marrow.
In this study the authors show how stem cells in the bone marrow are maintained by NPY via indirect regulation of the stromal microenvironment. By using different transgenic mouse models they show that NPY deficiency causes bone marrow impairments of HSC survival, as well as integrity of SN S nerve fibers and ECs. Pharmacological or endogenous NPY increase leads to HSC regeneration through the Y1 receptor. The fact that the authors divided their original paper into two papers improved very much the quality and focus of each paper. In addition, the authors invested a lot of hard work and managed to answer most of reviewer's comments. The molecular mechanistic aspects although improved, still need to be a bit fine tuned. Therefore I would like the authors to refer to the following comments.

We would like to thank the referee for the comments concerning our manuscript. As suggested by this referee's comments, we have carefully reconsidered and revised our manuscript.

Major comments
1. In a previous publication by the group of Nakauchi H (Cell, 2011, Yamazaki S et al) it was shown that non-myelinated schwann cells maintain stem cells in the BM niche by activating TGFb. Please cite this work as well as test if Schwann cells in WT mice express NPY receptor, which in turn will induce TGFb secretion.

According to the referee’s comment, we have also carefully investigated that whether schwann cells in WT mice express Y1 receptor. Schwann cells were prepared from the sciatic nerve of adult C57BL/6 mice as described (Nat Protoc. 2012, 7:1996-2004). Briefly, fresh sciatic nerves and the sciatic nerves that had been incubated in DMEM with 10% FBS, 1% penicillin/streptomycin and 0.25 mg/ml amphotericin B (Sigma) were rinsed with PBS, cut into 2-mm pieces and digested with a mixture of 0.05% collagenase (Sigma) at 37°C for 60 min. The digested materials were then centrifuged at 400g for 6 min and the supernatants were discarded. The pellets were resuspended in schwann cell culture medium and replated in poly-L-lysine and laminin coated dishes following cell counting. The dishes were incubated at 37°C under 5% CO2 for 3 weeks and culture medium was changed every 3 days. For identification of schwann cells, cells were stained GFAP (rabbit, Dako, N1506), S100b (rabbit, Dako, Z0311) and p75NGFR (rabbit, abcam, ab52987) (Supplementary Figure. 4A).
Next, we confirmed mRNA levels of Y1 receptor in schwann cells. The cultured adult schwann cells expressed Y1 receptors, although its expression was fewer than control macrophages (Supplementary Figure 4B). To investigate NPY-induced TGFb secretion in schwann cells, cells were treated with or without NPY (10 nM). The results showed no significant difference of TGF-b levels in NPY treated schwann cells and in CM derived from NPY treated schwann cells compared to control (Supplementary Figure 4C and D). These results indicated that TGF-b released by NPY-mediated Y1 receptor stimulation in schwann cells did not contribute to promote neuroprotection and HSCs survival in the BM. We have cited and commented about the previous study, and added these data as Supplementary Figure 4.

Supplementary Fig. S4 (related to Fig. 6). NPY induces TGF-b production though Y1 receptor in schwann cells. (A) Representative immunofluorescence adult schwann cells of GFAP, S100b and p75NGFR (green). Scale bar, 100 mm. (B) mRNA levels of Y1 receptor in schwann cells, control macrophage (positive control) and Y1 receptor depleted macrophage (negative control) (n = 4 per group). (C and D) Levels of TGF-b (C) in schwann cells and (D) in CM derived from schwann cells with or without NPY (n = 4 per group). *P < 0.05. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA.

2. The regulation of ECM embedded TGFb by osteoclasts and its role in Sca-1 bone forming progenitor cells was already shown. Please cite this paper and discuss it (X. Wu and Xu Cao in Cell Stem Cells 2010). 

As suggested by this referee’s comment, we have cited and commented about the previous study in discussion section. (p.21)

Minor comments
1. In supplementary fig. 3, the authors claim that the TGFb downstream pathway is activated. Please write what is this pathway.

   According to the referee’s comment, we have carefully reconsidered our manuscript. We have revised the sentence in the result section for clarification (p.16).

2. In the supplementary part quantitative PCR was done from isolated RNA from B...? Which cells the authors refer to?

   According to the referee’s comment, we have carefully reconsidered our manuscript. In supplementary figure 3, we investigated which factors released from macrophages induced neuroprotection. We screened and compared the conditioned medium (CM) of control or Y1 receptor deficient macrophages with and without NPY for 50 different secreted cytokines using an antibody-based mouse cytokine array (Supplementary Figure 3B). The CM of control macrophages with NPY revealed stronger signals in 4 array spots in comparison to the CM of control.
macrophages alone, such as IL20, TGF-b, TNF-a, TRAIL (Supplementary Figure 3C and D). We also confirmed the mRNA levels of these factors in macrophages through real-time PCR, and found up-regulated TGF-b expression in control macrophages with NPY treatment (Supplementary Figure 3E). However, Y1 receptor deficient macrophages with NPY treatment did not evaluate TGF-b expression both CM and macrophages. We have revised the result section for clarification (p.15).