THE
EMBO
JOURNAL

Manuscript EMBO-2010-75624

Neuronal CCL21 up-regulates microglia P2X4 expression and initiates neuropathic pain development

Knut Biber, Makoto Tsuda, Hidetoshi Tozaki-Saitoh, Keiko Tsukamoto, Emika Toyomitsu, Takahiro Masuda, Hendrikus Boddeke and Kazuhide Inoue

Corresponding author: Knut Biber, University of Freiburg

Review timeline:

Submission date:	10 August 2010
Editorial Decision:	25 August 2010
Revision received:	13 January 2011
Editorial Decision:	11 February 2011
Revision received:	28 February 2011
Accepted:	02 March 2011

Transaction Report:

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

1st Editorial Decision 25 August 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, reviewers 1 and 2 both express interest in your work, but both also raise significant concerns with the manuscript in its current form. Referee 3, while making no overall recommendation, also raises some important issues. The critical point here would be to provide stronger evidence that CCL21 specifically acts on activated microglia to induce P2X4 expression. As reviewer 2 notes, identifying the CCL21 receptor would be very important. Reviewer 1 also points out that excluding effects on T cells, that have been associated with neuropathic pain, would also be crucial.

Given the interest expressed by referees 1 and 2, I would like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I do realise that this will entail a significant amount of work, but I hope you find their comments constructive, and I stress that eventual acceptance of your manuscript would be contingent upon your satisfying the concerns particularly of the more critical referee 1. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the

© European Molecular Biology Organization

conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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 (Remarks to the Author):

The manuscript from Biber and co workers presents new data on the role of the chemokine CCL21 in the induction of neuropathic pain. The main results of this study are:
1/ After nerve injury, CCL21 is expressed and released by nociceptive sensory neurons and participate to the induction of neuropathic pain.
2/ CCL21 is likely to act by inducing microglial expression of the P2X4 receptor.

Overall this study provides novel insights on the mechanisms underlying neuropathic pain development, particularly regarding the involvement of CCL21. However several aspects of this study deserve the author's conclusions.

Major points:
In the CNS, several reports have proposed that endangered neurons express and release CCL21, which in turn promotes microglial activation. The data presented show that, following nerve injury, CCL21 expression is induced in a subpopulation of nociceptive neurons, yet CCL21 deficiency does not alter microglial activation (assessed by morphological criteria) but affect P2X4 receptor expression.
I'm concerned with the conclusion drawn by the authors. Although, IBA1 and OX42 immunostaining are clearly different between naive and injured plt mice, I'm not convinced that there are no differences in the activation state of microglia in injured WT and plt mice, at least from the data presented.
One possibility, which is somehow addressed in the discussion, is that CCL21 may control some specific states of microglial activation. I believe that this manuscript will be strengthened if a more precise analysis of the microglial activation state was performed in plt mice. The analysis of other parameters of microglial activation such as proliferation, quantification of the number of microglial cells, expression of specific makers, (P2Y12 receptors, Lyn kinase...) that are specifically induced in activated microglia need to be perform.

The author should also evaluate whether T cell recruitment in the spinal cord following nerve injury, is not altered in plt mice. This is of importance since subset of T cells are involved in the generation of neuropathic pain and plt mice present deficiencies in dendritic cell-mediated T cell induction.

Results depicted in figure 4b are very interesting and surprising. It is fascinating that a single intrathecal injection of CCL21 in nerved-injured plt mice is sufficient to established neuropathic pain. However the author made little effort to further characterized this effect. To me, the experiment shown in figure 4C is not really appropriate. It would have been difficult to interpret a different result (i.e. that CCL21 injection induces hypersensitivity in P2X4-KO mice). At least they should have investigated whether P2X4 expression is induced in microglia following CCL21 injection, but analysis of other microglial or T cell markers would also be necessary (see above comments). One possible interpretation of this experiment is that CCL21 provide a crucial triggering signal for further microglial activation and/or recruitment of other type of immune cells.

Other points:

© European Molecular Biology Organization

The reversal of hypersensitivity induced by CCL21 blocking antibody is rather small. Considering the result shown on figure 4b, it would have been interesting to see whether injection of the antibody prior and after nerve injury could have a much greater effect. In addition, it is unclear to me why hypersensitivity was only been measured at day 7 and not followed daily.

Figure 3b: CCL21-mediated P2X4 expression in primary culture of plt mice would have been an interesting control. In addition, what is the rational for using primary culture from rat microglia rather than from mice?

The method section is rather limited. There is no mention of the surgical procedures and absolutely no details about the different strain of mice used in this study (genetic background, age, sex...). Protocol regarding blocking antibody injection is also lacking.

Figure 1g is of poor quality. It seems to me that CCL21 staining is also present in dorsal horn cell bodies. Double immunohistochemistry experiment should be performed as a control.

Referee #2 (Remarks to the Author):

This is an interesting paper demonstrating the potential importance of the chemokine CCL21 in the genesis of neuropathic pain. The authors have previously published several papers demonstrating that CCL21 can be expressed in neurons and act as a neurotransmitter. That is the general theme in this paper as well in which CCL21 is expressed by neurons in the dorsal root ganglia. The work is mostly well performed and convincing. However,there is one major problem with the paper in that the authors make no attempt to localize the receptor for this chemokine in their studies and demonstrate that it is found in the appropriate place at the appropriate time. A number of papers indicate that astrocytes are positive for CCR7 and may be target of CCL21 (Gomez-Nicola et al., 2010). The authors of this MS in another paper suggest that the action is through CXCR3. Which receptor is present on microglial cells? There is absolutely no mention of CCR7 or CXCR3 in this MS. Why? The Allen Brain Institute images of adult mouse spinal cord does not exhibit either CXCR3 or CCR7. Are these receptors upregulated in the spinal cord after injury? In situ hybridization or immunohistochemical data on the appropriate spinal cord sections would solve this problem.

Other points.
There is no mention of how the injury was performed and what were the conditions used for behavioral testing. The reader is told that the injury is the chung model yet the authors use nomenclature associated with spared nerve injury (SNI). This may not seem important but they make the point that the CCL21 is present in L5 DRG but not L4 DRG. That is fine, but if they have severed the L5 spinal nerve (ala Chung model) then the behavior observed in this model is due to alterations in L4 and or L6 DRG. L5 neurons are no longer in contact with the peripheral targets.

It is also hard to believe that the injury (which severs nearly 90% of L5 DRG neurons) exhibits such a low percentage of injured cells that are positive for CCL21. What can be said about the CCL21 cells? Seems like the authors contend that these small neurons are TRPV1 positive but IB4 negative and CGRP negative. Most papers would contend that nearly all TRPV1 cells are CGRP-positive!

Microglia activation following peripheral nerve injury is known to be present in both dorsal and ventral horns. How do so few cells releasing a factor activate cells in both portions of spinal cord?

Referee #3 (Remarks to the Author):

Major concerns

While tactile allodynia is a component of neuropathic pain, at least one other measure should be tested in key experiments.

It is imperative that a non injurious noxious stimulus be used as a control, for example, electrical stimulation of a peripheral nerve or inflammation rather than nerve injury.

Why are thermal rather than mechanical tests of nociception reported as controls in the plt mice?

Given the recent failure of minocycline in a clinical trial, some doubt is cast on the clinical importance of the proposed mechanism.

It is unclear why PWT takes so long (48 hours) to recover in P2X4-deficient mice.

1st Revision - authors' response 13 January 2011

Referee #1

Major points:
In the CNS, several reports have proposed that endangered neurons express and release CCL21, which in turn promotes microglial activation. The data presented show that, following nerve injury, CCL21 expression is induced in a subpopulation of nociceptive neurons, yet CCL21 deficiency does not alter microglial activation (assessed by morphological criteria) but affect P2X4 receptor expression.
I'm concerned with the conclusion drawn by the authors. Although, IBA1 and OX42 immunostaining are clearly different between naive and injured plt mice, I'm not convinced that there are no differences in the activation state of microglia in injured WT and plt mice, at least from the data presented.
One possibility, which is somehow addressed in the discussion, is that CCL21 may control some specific states of microglial activation. I believe that this manuscript will be strengthened if a more precise analysis of the microglial activation state was performed in plt mice. The analysis of other parameters of microglial activation such as proliferation, quantification of the number of microglial cells, expression of specific makers, (P2Y12 receptors, Lyn kinase...) that are specifically induced in activated microglia need to be perform.

Author's response:
We completely agree with the referee at this point. This comment makes it obvious that our discussion concerning the differences in microglia activity in WT and plt mice was not sufficiently clear and we would like to thank the referee to point this out.
We followed the suggestion of the referee and provide now a more detailed analysis of activated microglia in WT and plt animals after nerve lesion. We now determined microglia proliferation phosphorylated histone H3 (p-HisH3), quantified microglia cell numbers and morphology (Iba-1 staining) and analysed the expression of Lyn kinase. As shown now in the revised figures 3 and 4, there are no major differences for OX-42, p-HisH3, Lyn kinase and Iba1 for the microglia between WT and plt animals after spinal nerve injury. These data thus corroborate our conclusion that CCL21 is specifically required for P2X4 up-regulation and not necessarily related to other parameters of microglia activation.

We have furthermore changed the appropriate parts in the discussion (page 13-14), where it is now stated that, although morphologically indistinguishable activated microglia in WT and plt mice reflect functionally different states.

The author should also evaluate whether T cell recruitment in the spinal cord following nerve injury, is not altered in plt mice. This is of importance since subset of T cells are involved in the generation of neuropathic pain and plt mice present deficiencies in dendritic cell-mediated T cell induction.

Authors response: The referee is right, it is known from the plt model that these animals display different T cell response. We therefore have analysed the T cell infiltration of the spinal cord in response to nerve injury by CD3 staining. It now is shown in supplementary figure 3 that only few T cells can be observed in the spinal cord after nerve injury and that there are no differences in T cell numbers between WT and plt animals.

Results depicted in figure 4b are very interesting and surprising. It is fascinating that a single intrathecal injection of CCL21 in nerved-injured plt mice is sufficient to established neuropathic pain. However the author made little effort to further characterized this effect. To me, the experiment shown in figure 4C is not really appropriate. It would have been difficult to interpret a

© European Molecular Biology Organization

different result (i.e. that CCL21 injection induces hypersensitivity in P2X4-KO mice). At least they should have investigated whether P2X4 expression is induced in microglia following CCL21 injection, but analysis of other microglial or T cell markers would also be necessary (see above comments). One possible interpretation of this experiment is that CCL21 provide a crucial triggering signal for further microglial activation and/or recruitment of other type of immune cells.

Authors response: We have addressed this suggestion and now show that microglia in plt animals up-regulate P2X4 expression in response to CCL21 injection. Since P2X4 receptor expression in microglia was the most prominent difference between WT and plt mice we observed (see comment above), we did not further investigate microglia morphology or T cell infiltration. We, however, further show that CCL21 stimulation of cultured microglia causes an up-regulation of P2X4 receptor expression, an effect that was also found in microglia from plt mice (please see revised figure 3). Based on these data we suggest that CCL21 is the factor that is responsible for P2X4 receptor induction in microglia after nerve injury. To further investigate this direct relationship between CCL21 and P2X4 it was of importance to investigate the potential effects of CCL21 injection in P2X4 receptor deficient animals. The data now shown in figure 5D confirm that CCL21 injection in the absence of P2X4 does not have a pain-inducing effect, ergo CCL21 requires P2X4 receptor function in the cascade leading to neuropathic pain. Other pathways are most likely not involved which is the main message from this experiment. Although we agree with the referee that another result would have been very difficult to interpret, we still feel that it was important to perform this experiment that controls for the pathway that we suggest.

Other points:
The reversal of hypersensitivity induced by CCL21 blocking antibody is rather small. Considering the result shown on figure 4b, it would have been interesting to see whether injection of the antibody prior and after nerve injury could have a much greater effect. In addition, it is unclear to me why hypersensitivity was only been measured at day 7 and not followed daily.

Author's response: We now have repeated the antibody experiment and now treated with CCL21-neutralizing antibody twice a day from day 0 (15 min before nerve injury) to postoperative day 3. The hypersensitivity was assessed throughout the 14 days of testing periods and the results are now shown in revised figure 2E. We found that the antibody treatment was sufficient to inhibit the formation of neuropathic pain almost completely for the first 7 days. At day 14 only a slight pain reaction was detectable. These results suggest that the inhibition of CCL21 function early after nerve injury might be a valuable drug target to prevent the formation of neuropathic pain.

Figure 3b: CCL21-mediated P2X4 expression in primary culture of plt mice would have been an interesting control. In addition, what is the rational for using primary culture from rat microglia rather than from mice?

Authors response: We agree with the referee and include now in the revised figure 3 also effects of CCL21 on mouse (WT and plt) microglia P2X4 receptor expression. It is now shown that all microglia respond to CCL21 with a similar up-regulation of P2X4 receptor expression. The rational for using rat microglia in the first place was the lack of an appropriate mouse P2X4 receptor antibody. This antibody only became available at latter stages of our study.

The method section is rather limited. There is no mention of the surgical procedures and absolutely no details about the different strain of mice used in this study (genetic background, age, sex...). Protocol regarding blocking antibody injection is also lacking.

Authors response: According to the suggestion of the referee we provide more details about the used methods (Page 15-18)

Figure 1g is of poor quality. It seems to me that CCL21 staining is also present in dorsal horn cell bodies. Double immunohistochemistry experiment should be performed as a control.

Authors response: This comment of the referee might be due to the small size of the images at rather low resolution. In the revised Figure 1G, we have now replaced them with bigger images showing CCL21 immunofluorescence data at a higher resolution. As requested by the referee, we have now performed double-immunolabeling of CCL21 with the microglial marker OX-42 and found that

© European Molecular Biology Organization

CCL21 immunofluorescence shows a fibrous morphology and does not overlapped with OX-42 immunofluorescence. We never observed CCL21 staining in cell bodies. A high magnification image of double immunofluorescence has now been incorporated into the manuscript as Supplementary Figure 1.

Referee #2 (Remarks to the Author):

This is an interesting paper demonstrating the potential importance of the chemokine CCL21 in the genesis of neuropathic pain.The authors have previously published several papers demonstrating that CCL21 can be expressed in neurons and act as a neurotransmitter.That is the general theme in this paper as well in which CCL21 is expressed by neurons in the dorsal root ganglia.The work is mostly well performed and convincing.However,there is one major problem with the paper in that the authors make no attempt to localize the receptor for this chemokine in their studies and demonstrate that it is found in the appropriate place at the appropriate time. A number of papers indicate that astrocytes are positive for CCR7 and may be target of CCL21 (Gomez-Nicola et al., 2010). The authors of this MS in another paper suggest that the action is through CXCR3. Which receptor is present on microglial cells? There is absolutely no mention of CCR7 or CXCR3 in this MS. Why? The Allen Brain
Institute images of adult mouse spinal cord does not exhibit either CXCR3 or CCR7. Are these receptors upregulated in the spinal cord after injury? In situ hybridization or immunohistochemical data on the appropriate spinal cord sections would solve this problem.

Authors response: In agreement with the referees suggestion, we have performed various experiments to identify the receptor for CCL21. As pointed out by the referee there are two potential receptors for CCL21 in mice: CXCR3 and CCR7. We have performed quantitative PCR for both receptors in spinal cord tissue of mice subjected to nerve injury. As shown now in revised figure 6 we observed CXCR3 mRNA expression, but CCR7 mRNA expression was not reliably detectable. Neither mRNA was regulated by nerve injury. These findings are in complete agreement with previous findings from our group and others. We have shown that microglia and astrocytes(Biber et al., 2001; Biber et al., 2002; Rappert et al., 2002, 2004; Dijkstra et al., 2004; de Haas et al., 2007) express CXCR3 mRNA, protein and respond to CXCR3 ligands functionally. In brain tissue CCR7 mRNA is generally not found as demonstrated for cultured neurons, astrocytes and microglia (Biber et al., 2001) or brain tissue (Dijkstra et al., 2006). Nor is CCR7 protein detectable by flow cytometry in acutely isolated microglia (de Haas et al., 2007). Neither cultured astrocytes nor microglia respond to the CCR7 chemokine ligand CCL19 (Rappert et al., 2002; van Weering et al., 2010), thus there are various lines of evidence that CCR7 is not expressed in unchallenged brain tissue or brain cell cultures. The referee refers to the recent paper Gomez-Nicola et al., 2010, where it is suggested that astrocytes express CCR7. To our knowledge this is the only paper that would state this. We would like to remark here that this evidence is based on antibody staining only. No mRNA data or functional analysis would corroborate this conclusion. The first author of the current study (Knut Biber) is now working for 10 years on CCL21 receptors in glia cells and has tried all commercially available CCR7 antibodies. None of them was found reliable, since the staining pattern was indistinguishable in controls and CCR7 deficient tissue. The Gomez-Nicola paper does not describe such a thorough control for their antibody used, which is why we consider the data published about CCR7 expression in astrocytes as difficult to interpret.
We and other have provided evidence that functional CCR7 expression in microglia can be induced by TLR activation in microglia (Dijkstra et al., 2006, Takahashi et al., 2005) however, this is not the case in the spinal cord after nerve injury (revised figure 6). Our PCR analysis would suggest that based on the expression level, CXCR3 but not CCR7 may be a receptor for CCL21 in the spinal cord. In order to further elucidate this nerve injury experiments where performed with CXCR3 and CCR7 deficient animals as shown now in the manuscript (revised figure 6). To our surprise none of the receptor knockouts displayed a clear phenotype indicating that neither CXCR3 nor CCR7 is solely responsible for the effects of CCL21 in the spinal cord. We subsequently stimulated cultured microglia with specific ligands for CXCR3 (CXCL10) and CCR7 (CCL19). None of them induced the expression of P2X4 in microglia in contrast to CCL21 (revised figure 6). Taken together these data may suggest the expression of yet unidentified CCL21 receptor in microglia, corroborating earlier findings of our group (van Weering et al., 2010).

Other points.

© European Molecular Biology Organization

There is no mention of how the injury was performed and what were the conditions used for behavioral testing. The reader is told that the injury is the chung model yet the authors use nomenclature associated with spared nerve injury (SNI). This may not seem important but they make the point that the CCL21 is present in L5 DRG but not L4 DRG. That is fine, but if they have severed the L5 spinal nerve (ala Chung model) then the behavior observed in this model is due to alterations in L4 and or L6 DRG. L5 neurons are no longer in contact with the peripheral targets.

Authors response: As suggested by the referee we now have described the methods in detail in the revised text (page 15-18). In agreement with the referees suggestion the misleading abbreviation "SNI" was removed from the manuscript. The referee comments about the nerve lesion model and states correctly that the behaviour in response to mechanical stimulation after nerve lesion might be mediated through L4 or L6 DRG neurons. However, the L4 and L6 spinal nerve projects not only to the L4 and 6 segment, respectively but also the neighbouring segments L5 where the most dramatic activation of microglia is seen. Thus, L4 and L6 DRG neurons are not damaged, but the sensory information transmitting through these neurons is altered by P2X4 expressing microglia at the level of the dorsal horn (Tsuda et al., 2003; Coull et al., 2005).

It is also hard to believe that the injury (which severs nearly 90% of L5 DRG neurons) exhibits such a low percentage of injured cells that are positive for CCL21. What can be said about the CCL21 cells? Seems like the authors contend that these small neurons are TRPV1 positive but IB4 negative and CGRP negative. Most papers would contend that nearly all TRPV1 cells are CGRP-positive!

Authors response: The referee comments on the type of DRG neurons that are CCL21 positive. To address this comment we have quantified the co-localization of TRPV1 and CGRP in L5 DRG sections from wild-type mice by double-immunostaining and found that about 40 % of TRPV1-positive neurons are double-labelled with CGRP (please refer to the provided figure). These findings are consistent with several reports from others (Cell Tissue Res 323: 27-41, 2006; Brain Res 1219: 59-65, 2008). In addition, the percentage of the co-localization is not changed after L5 spinal nerve injury. Why other publications find that nearly all TRPV1 cells are CGRP-positive can not be explained here and might be due to species differences or different antibodies used.
However, it is known that neurons are highly specialized cells with different functions and different properties. Even very small neuronal units like the DRGs contain different types of neurons, as can already be seen by their different sizes. Our findings that the L5 spinal injury induces CCL21 expression in a specific neuronal subtype only would very much fit to this general concept, showing that not all DRG neurons respond to a given stimulus (nerve lesion) the same way. It remains unclear why only TRPV1-positive small-sized DRG neurons respond to injury with CCL21 expression and it will of interest in the future to explore the underlying mechanisms. However, this will require a complete study on its own and thus we consider this beyond the scope of the present, initial report on the role of CCL21 in nerve injury-induced tactile allodynia.

Microglia activation following peripheral nerve injury is known to be present in both dorsal and ventral horns. How do so few cells releasing a factor activate cells in both portions of spinal cord?

Authors response: We agree with the referee. CCL21 can not be the only factor that is released in order to activate microglia, in fact our data clearly show the involvement of other factors since in plt mice we find a rather robust morphological activation of microglia in the dorsal horn (revised figure 3 and 4). We now discuss this point in detail on page 12. The referee also refers to microglia activation in the ventral horn after injury. There are numerous cell bodies of dying cells in the ventral horn in response to nerve injury (motorneurons are dying here) and microglia are known to rapidly respond to neuronal cell death. Dying cells release numerous factors for example purines, CX3CL1, or TLR ligands. Thus in the absence of CCL21, which was never observed in dying motorneurons, multiple other signals could account for the activation of microglia in the ventral horn of the spinal cord.

Referee #3 (Remarks to the Author):

Major concerns
While tactile allodynia is a component of neuropathic pain, at least one other measure should be tested in key experiments.

Authors response: The referee is correct tactile allodynia is not the only component of neuropathic pain also thermal hypersensitivity belongs to neuropathic pain sensations. As suggested by the referee, we have tested heat hypersensitivity in our model. Spinal nerve injury decreased the latency to respond (either lick or shake the hindpaw) by heat stimulus. The enhanced responsiveness to a heat stimulus was comparable between WT and plt mice. This finding suggests that plt mice are resistant preferentially to tactile allodynia development, rather than thermal hyperalgesia. We have now incorporated the data into the revised Fig. 2B and corresponding text.
We would like to add here that the aim of the present study was to elucidate the mechanisms underlying the most troublesome and refractory aspects of nerve injury-induced pain hypersensitivity which is tactile allodynia (Woolf & Mannion, Lancet 355, 1959-1964, 1999). Thermal hypersensitivity is not a common feature of neuropathic pain in humans (e.g. Scholz & Woolf, Nat. Neurosci. 5: 1062-1067, 2002) and in contrast to tactile allodynia is thermal hypersensitivity treatable by narcotics like morphine (Arner & Meyerson, Pain 33: 11-23, 1988; Woolf & Mannion, Lancet 355: 1959-1964, 1999; Woolf, Neurobiol. Dis. 7: 504-510, 2000). These differences in morphine response between tactile allodynia and thermal hypersensitivity has also been described in the mouse model used in our study (Lashbrook et al., Pain 82: 65-72, 1999; Wegert et al., Pain 71: 57-64, 1997). Finally, tactile allodynia following spinal nerve injury is longer-lasting and is a more robust feature of the nerve-injury model than is heat hypersensitivity (Kim & Chung, Pain 50: 355-363, 1992). All these data indicate that tactile allodynia and thermal hypersensitivity are due to different mechanisms and are thus in agreement with our findings that CCL21 is required for tactile allodynia development but not for thermal hypersensitivity. We have now discussed this point in the manuscript (page 11).

It is imperative that a non injurious noxious stimulus be used as a control, for example, electrical stimulation of a peripheral nerve or inflammation rather than nerve injury.

Authors response: The referee is correct. It is important to use non injurious stimuli as controls. We have included such controls in revised figure 2 of the manuscript. It is shown that WT and plt mice did not differ in heat sensitivity or mechanical stimulation, indicating that there is no general pain sensing deficit in plt animals. Since this is most likely due to normal neuronal function an electrical stimulation was not done. Since inflammatory pain is caused by different mechanisms than neuropathic pain after spinal nerve injury this would be a project on its own and outside of the scope of this manuscript that focuses on spinal nerve injury.

Why are thermal rather than mechanical tests of nociception reported as controls in the plt mice?

Authors response: As outlines above, we now have performed a mechanical test of nociception. We applied 2.0 g of von Frey filament to the mouse hindpaw ten times intermittently and found that the paw withdrawal frequency is indistinguishable between WT and plt mice. The result indicate that plt animals have no general pain sensing deficit and these results have now been incorporated into the revised Fig. 2D and corresponding text.

Given the recent failure of minocycline in a clinical trial, some doubt is cast on the clinical importance of the proposed mechanism.

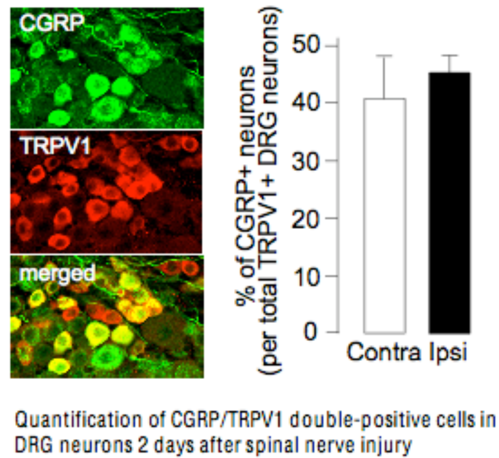
Authors response: Minocycline is an antibiotic that is at the moment considered to be an inhibitor of microglia activity. However, it is not understood at all how minocycline affects microglia function. Since our data show that microglia activity in the development of neuropathic pain is not due to an ON or OFF situation, minocycline may inhibit microglia actions that are not related to neuropathic pain. Therefore, in order to develop better drugs for this pain condition it is required to in detail understand the cascade that leads to neuropathic pain. In this respect our data may be a valuable contribution showing that CCL21 may be a potential drug target to prevent the development of neuropathic pain.

It is unclear why PWT takes so long (48 hours) to recover in P2X4-deficient mice.

Authors response: We are not sure what the referee would like to ask here. There is no statistically significant difference in the recovery of PWT between P2X4-deficient animals (revised figure 5 B) and contra lateral side in plt animals (revised figure 5D). As discussed in the manuscript, the mechanisms behind this temporary hypersensitivity in response to CCL21 injection are not

understood at the moment and require further work. It therefore is very difficult to comment on this question.

Figure for referee



Quantification of CGRP/TRPV1 double-positive cells in DRG neurons 2 days after spinal nerve injury

2nd Editorial Decision 11 February 2011

Thank you for submitting your revised manuscript to the EMBO Journal.

The original referee #1 and 2 has now seen your manuscript and their comments to the authors are provided below. As you can see both referees are pleased with the introduced changes. Referee #1 has a few remaining concerns that should be resolved before acceptance here. I would therefore like

to ask you to respond to the remaining concerns in a final revision.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

I look forward to seeing the final version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This revised version of the manuscript by Biber et al. is greatly improved. The authors answered to most of the criticisms raised.

However, I still have a few concerns.
Regarding the activation state of microglia in plt-/- mice, there is a strong discrepancy between p-HistH3 and IbaI immunostaining after spinal nerve lesion (Fig. 4). Clearly, the number of cell p-HistH3 positive is very low compared to the number of microglia. In addition while most of the microglial cells are located in lamina I and II, the few histone positive cells are scattered in the whole quadrant.
It is also difficult to compare staining performed at 2 days post lesion (p-HistH3) versus 7 days (Iba1).
Finally, it is not clear why the authors used p-HistH3 staining to assess microglia proliferation rather than KI-67,which is often used and better characterized in terms of microglial proliferation.
Similarly, because of the long process of microglia, counting IbaI positive cells can be misleading in the absence of nuclei counter staining.
I believe that these experiments could be improved.

My second concern is related to the reversing effect of CCL21-blocking antibody. One hypothesis proposed by the authors is that CCL21 is the signal triggering P2X4 expression in microglia. As such they demonstrate that CCL21-blocking antibody when administered around the time of the lesion can prevent the development of hypersensitivity. It would be of interest to know whether the neutralizing CCL21 antibody is still efficient once hypersensitivity is fully installed. This could be achieved by the same instillation protocol as described in Figure 2E starting at day seven and hypersensitivity assessment at day 14. I believe that such an experiment would hold a real therapeutic interest.

Very minor point
For clarity, In figure 5C, a mention of protein immunostained (here P2X4) should appear.

Referee #2 (Remarks to the Author):

This manuscript describes a potential role for the chemokine CCL21 in the induction of neuropathic pain. My previous major problem with the paper had been that, although the authors had described the expression of the ligand CCL21, there was no evidence as to its potential receptor being present under these conditions in the spinal cord.
The authors have now conducted additional studies in which they demonstrate that CXCR3, a potential receptor for CCL21 is expressed, whereas CCR7,a second potential receptor, is not expressed. Hence, the situation is now much clearer and the manuscript is much more complete. The authors also answered my other questions concerning their pain measurements etc.

Consequently, at this point, I am happy to enthusiastically recommend publication of this paper.

2nd Revision - authors' response 28 February 2011

1) Referee one

Regarding the activation state of microglia in plt-/- mice, there is a strong discrepancy between p-HistH3 and IbaI immunostaining after spinal nerve lesion (Fig. 4). Clearly, the number of cell p-HistH3 positive is very low compared to the number of microglia.

Our answer) We agree with the referee that the number of p-HistH3-positive microglia is low. Since the duration of microglia mitosis *in vivo* has yet not been determined, a clear explanation for this low number can currently not be provided. It might be possible that the low number of p-HistH3 microglia is due to a rather short duration of mitosis resulting in a rather short time where the epitope can be recognized by the antibody. In another current project we observed the same results in the dorsal horn of rats after nerve injury (Tsuda et al., Brain, in press) and are thus convinced that the low number of p-HistH3-positive microglia is not an artifact. This point has now shortly been included into the manuscript (Page 7, second paragraph)

2) Referee one

In addition while most of the microglial cells are located in lamina I and II, the few histone positive cells are scattered in the whole quadrant.

Our answer) The referee addresses here an unknown issue, since it is yet not understood why on day 7 after spinal nerve injury dorsal horn microglia are accumulated in lamina I and II, whereas at earlier time points microglia proliferate also on other regions of the dorsal horn. Our hypothesis would be that the change in laminar distribution of microglia may result from migration of dorsal horn microglia into lamina I and II. Such a reaction of microglia is known for example in the hippocampus, where in response to entorhinal cortex lesion the resulting accumulation of activated microglia in the mid-molecular layer of the hippocampus is due to both proliferation and migration of microglia. Since next to CCL21 (our paper) also other chemokines (CCL2 and CX3CL1) are induced in the spinal cord after peripheral nerve lesion, all chemokines could very well account for the attraction of microglia from other parts. This question is at the moment under detailed investigation in our laboratory.

A short statement concerning this issue has now been included into the manuscript (Page 12, last paragraph)

3) Referee one

It is also difficult to compare staining performed at 2 days post lesion (p-HistH3) versus 7 days (Iba1).

Our answer) This comment of the referee shows that we were not completely clear in our results description. We agree that it would be at least difficult (if not scientifically incorrect) to compare stainings for different epitopes in tissue at different time points and to draw conclusions from that. We therefore (as stated on page 7 of the manuscript) analyzed spinal cord tissue at 2, 7 and 14 days for all markers. For none of these markers, a difference between wild type and plt animals was found. In order not to show too many negative data, we exemplified our finding and show p-HistH3 as a marker of mitosis 2 days after the injury, whereas counting the number of Iba1+ microglia on day 7 is shown to exemplify that the increase in number of microglia as a consequence of proliferation is not different between WT and plt mice too.

© European Molecular Biology Organization

To clarify this issue we now describe this in detail (page 7, second paragraph) and provide representative stainings for all markers (p-HistH3, Iba1 and LynK) for the complementary time points in supplementary figure 3.

4) Referee one
Finally, it is not clear why the authors used p-HistH3 staining to assess microglia proliferation rather than KI-67, which is often used and better characterized in terms of microglial proliferation.
Our answer) We agree that Ki-67 is also a good marker of proliferation and we have used this marker to determine microglia proliferation in rats before. For the current manuscript we tried Ki-67 staining without satisfying results in the mouse spinal cord. Whether or not this might be due to a problem in the lot of the Ki-67 antibody we used is not known and we could not solve this point in the limited time that we had to prepare the resubmission. Since in our hands p-HistH3 was a working maker for mouse microglia proliferation we continued our analysis with this antibody.

5) Referee one
Similarly, because of the long process of microglia, counting IbaI positive cells can be misleading in the absence of nuclei counter staining.
Our answer) The referee is correct, microglia have very long processes, which one need to take into account by quantifying microglia numbers. Since Iba1 can clearly labels microglial cell bodies and the processes, and since the shape of Iba1-labeled microglial cell bodies is very much different from the processes, it is easily possible to distinguish between cell bodies and processes in Iba1 stained microglia without the need for nuclei counter staining. Please be aware of the fact that this is in stark contrast to OX-42 which mainly labels the processes of microglia cells. For the here provided quantification of microglia numbers only cell bodies where counted and we are convinced that a re-quantification with nuclear counter staining would reveal the same results.

Referee one
I believe that these experiments could be improved.
Our answer) The referee did not suggest specific experiments here, and we feel that all above mentioned concerns have been addressed in the text without additional experimentation.

My second concern is related to the reversing effect of CCL21-blocking antibody. One hypothesis proposed by the authors is that CCL21 is the signal triggering P2X4 expression in microglia. As such they demonstrate that CCL21-blocking antibody when administered around the time of the lesion can prevent the development of hypersensitivity. It would be of interest to know whether the neutralizing CCL21 antibody is still efficient once hypersensitivity is fully installed. This could be achieved by the same instillation protocol as described in Figure 2E starting at day seven and hypersensitivity assessment at day 14. I believe that such an experiment would hold a real therapeutic interest.
Our answer) The referee suggests here a new experiment, that was not suggested in the first round of review. We also have initially thought about this experiment, discussed it again intensively in the first revision round and finally decided not to include it for the following reasons. First, it was our aim to investigate the potential role of CCL21 in the initiation of neuropathic pain and not its maintenance. Second, our antibody treatment data would argue against an important role of CCL21 at later time points after the lesion, since there was no further increase in pain scores for 12 days after the antibody treatment was stopped. Thirdly, as shown in Figure 1 CCL21 expression in neurons peaks at 24 hours after the lesion and diminishes at later time points. We never investigated CCL21 expression at 7 days after nerve lesion in detail, so we do not know whether CCL21 is still expressed at this time point. So the suggested experiment of treating animals with CCL21 antibody

at 7 days after the lesion onwards might produce negative data, which would be difficult to include into the current manuscript. We completely agree with the referee that any treatment that would help to diminish already ongoing neuropathic pain would hold a real therapeutic interest. Such treatments could be blocking of microglial P2X4 function (Nature 424, 778-783, 2003). Due to the above mentioned reasons CCL21 might not be the right target here. We see the therapeutic interest of CCL21 from a different angle. The pathway presented in our manuscript might offer the possibility to prevent the development of neuropathic pain, for example in patients that come to the hospital with an acute peripheral nerve injury.

After consideration with the editorial office of EMBO J we now discuss our view about the therapeutic potential of CCL21 in the discussion on page 14, second paragraph.

Very minor point
Moreover, figure 5 was changed according to the suggestion of the referee.

Acceptance Letter 1 March 2011

Many thanks for submitting the final version of your manuscript. I have now had the chance to look through it and everything looks fine. I am pleased to be able to tell you that we can now accept the manuscript for publication - you should receive the formal acceptance message shortly.

Thanks for choosing EMBOJ for publication of this study, and congratulations on a fine piece of work!