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Author/s:

Goodings, L;He, J;Wood, AJ;Harris, WA;Currie, PD;Jusuf, PR

Title:

In vivo expression of Nurr1/Nr4a2a in developing retinal amacrine subtypes in zebrafish Tg(nr4a2a:eGFP) transgenics

Date:

2017-06-01

Citation:

Goodings, L., He, J., Wood, A. J., Harris, W. A., Currie, P. D. & Jusuf, P. R. (2017). In vivo expression of Nurr1/Nr4a2a in developing retinal amacrine subtypes in zebrafish Tg(nr4a2a:eGFP) transgenics. *Journal of Comparative Neurology*, 525 (8), pp.1962-1979. <https://doi.org/10.1002/cne.24185>.

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**** NOTE **** - The decision letter and review of the previous draft and the authors' response letter are included below. Please consider them in your evaluation of the revised draft.

PREVIOUS DRAFT DECISION LETTER AND REVIEWS:

13-Nov-2016

Dear Dr. Jusuf:

Your manuscript,

JCN-16-0184 In vivo expression of Nurr1 / Nr4a2a in developing retinal amacrine subtypes in zebrafish Tg(nr4a2a:eGFP) transgenics,

has now been reviewed by our scientific referees and the comments are enclosed.

Although this manuscript addresses a worthwhile topic, the work will require substantial revision before it can be reconsidered for publication. The reviewers point out numerous issues; some can be resolved simply while others may require additional experiments.

Of particular concern is the lack of documentation of antibody specificity in fish. Simple reference to manufacturing specifications is not sufficient unless the company has tested the antibody against fish materials. A Western blot of mammalian tissues gives little indication of whether a cross-reacting protein may exist in zebrafish or other related teleosts. Please refer to our antibody reporting policies. This represents an essential revision.

The reviewers also offer numerous other points in need of attention. We hope that these detailed reviews offer sufficient guidance on how to move forward with this work.

Also, for the convenience of the reviewer and editors, may we ask that you use the Track Changes function in Word, or a similar word-processing function, to highlight the changes you make in your revised resubmission. In addition, please upload a clean copy of your revised text, in which the change tracking feature is turned off, as some reviewers prefer using that for their evaluation.

If you submit a revision of this paper, please explain in your cover letter how you have changed the present version. Re-review by a single external referee will be required. Please note that Journal of Comparative Neurology policy requires that the manuscripts for single re-review be returned within three months. If you require longer than three months, your paper will require re-review by a second reviewer. Please follow the instructions for resubmission in Journal of Comparative Neurology Guide for Authors.

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Thank you for your submission to Journal of Comparative Neurology.

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version record](#). Please cite this article as [doi:10.1002/cne.24185](https://doi.org/10.1002/cne.24185).

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Sincerely,

Dr. Thomas Finger
Associate Editor, Journal of Comparative Neurology

Dr. Patrick Hof
Editor-in-Chief, Journal of Comparative Neurology

You can upload your revised manuscript and submit it through your Author Center. Log into <https://mc.manuscriptcentral.com/jcn> and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions."

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) in the space provided. You can use this space to document any changes you make to the original manuscript.

IMPORTANT: We have your original files. When submitting (uploading) your revised manuscript, please delete the file(s) that you wish to replace and then upload the revised file(s). Please note that submitting a revision of your manuscript does not guarantee eventual acceptance.

Reviewer: 1

Comments for the Authors

This paper describes a solid investigation of the expression of Nr4a2a in the zebrafish retina. It is a carefully done set of experiments and is well interpreted and put into context of previous results. I have some minor concerns.

1. I am sure it is much easier to appreciate the co-labelling with various markers under the microscope than on the page or computer screen. I think it is possible, but a struggle, to see some of the points the authors are making on the figures I looked at. I would advise the authors to brighten up the figures and to do even larger enlargements of some of the key findings so that readers do not have to squint to see things.
2. The grammar is a little loose in places. Some careful editorial work or a good grammar checker is essential.

Reviewer: 2

Comments for the Authors

Overview:

Amacrine cells are retinal interneurons that lie at the border between the inner nuclear layer and the inner plexiform layer. As many as 30 different dendritic branching patterns distinguish amacrine cell subtypes. In the late 70's and early 80's, with the development of immunohistochemistry, many of these subtypes were found to have unique immunogenicity, with some reactive for substance P, others for parvalbumin, and so forth. Morphological classification was confirmed by selective immunoreactivity, and in many cases, these patterns could be seen across species, confirming not only the legitimacy of the morphological subtypes, but also the cross-species specificity of antibodies. The manuscript of Goodings et al examines the origins of these different antigenic types during retinal development in zebrafish. They test the important idea that development of amacrine subtypes follows a time sequence in activation of transcription factors. In particular, they follow the tyrosine hydroxylase immunoreactive subtype, which follows the

sequence Atoh7=>PTf1a=>Nr4a2a=>tyrosine hydroxylase. Among cells following this sequence, tyrosine hydroxylase is the only immunoreactive peptide found in a screen of 8 peptides known to be selective for amacrine types. To be sure, not all amacrines following the sequence Atoh7=>Ptfia=>Nr4a2a produced tyrosine hydroxylase. About half of these were simply 'GABAergic'. Perhaps further transcription factors in the sequence leading to tyrosine hydroxylase immunoreactivity are yet to be found. The intellectual content of this research line is very exciting. Since this is an antibody paper, the reviewer needs to consider the application of the Clifford Saper JCN editorial 'An Open Letter to Our Readers on the Use of Antibodies'. This elegant editorial details the stringency with which one may be certain that an antibody recognizes an epitope, with the highest certainty being that mutants for the epitope are unreactive, but pointing out that even in this case, one can't be certain that the antibody wasn't reactive with a downstream product. The current study uses antibodies characterized in rodents to label zebrafish. The issue needs to be addressed. Though the Saper editorial does not mention this situation, the reviewer thinks that a characterized antibody that labels an amacrine subtype in both rodents and zebrafish is a reasonably stringent criterion for antibody specificity in the uncharacterized species. Nonetheless, for tyrosine hydroxylase, there are two paralogues th1 and th2 in zebrafish. Perhaps the authors should consider a test of antibody reactivity for tyrosine hydroxylase in a morpholino knockout of th1. In any case the TH paralogue issue should be addressed. The Saper editorial also does not deal with criteria for credibility of transgenic reporter lines. One of the achievements of this manuscript is a transgenic reporter line for Nr4a2a. The authors have compared the reporter eGFP fluorescence to the developmental sequence of in situ hybridization for Nr4a2a mRNA. This was carefully done, and the transgene reporter line is credible.

While the thematic material of the manuscript is important, the text is plagued by syntax problems, some of which make idea structure difficult to parse. Journals don't supply copy editors any more. The authors will need to find their own. The 'Conclusions' section was strange, reading more like a prospectus than the product of experimentation.

Specific comments:

The authors use the words 'perdure, perdurant, and perdurance' 13 times in manuscript and legend. Presumably these refer to the 'half-life of eGFP'. While the word is very cool, it's extensive use tends to beg the question, what is the eGFP half-life? Are adult retinas fluorescent in the transgenic, and if so, in which cells and regions? That is the problem with 'perdure' so perdurantly. Otherwise it is completely reasonable to expect that eGFP need not overlap either the time or subcellular localization of the native Nr4a2a protein.

Abstract:

'The context of Nr4a2 expression (which cells when within which gene networks) and function remains poorly understood.' Fix the sentence.

'Nr4a2a:eGFP is confined to a specific progenitor lineage identified by sequential expression of atonal bhlh transcription factor 7 and pancreas transcription factor 1 a, which primarily drive ganglion and amacrine fates respectively.' This MS shows atoh7 followed by PTf1a drives an amacrine cell fate. Ganglion cell fates are less well defined, but start with atoh7, and probably sometimes followed by Nr4a2a, as seen in the MS. The sentence is unclear because it tries to do too much.

Methods

'Wholemouts embryos were dehydrated serially' 'wholemount embryos'

Results

'A detailed temporal time series were conducted to follow the onset and perdurance of the eGFP signal' 'was conducted'

'The rabbit anti-secretagogin antibody (RRID: AB_203 4060) used shows identical staining to a commercial and non-commercial secretagogin antibody' It sounds like the authors tried 3

secretagogin antibodies, alternatively they are quoting results obtained elsewhere. Please elaborate.

In Fig. 1, as in Fig. 2, the authors might want to include the column headers 'dorsal' and 'lateral'. Results header: 'Nr4a2a:eGFP is expressed post-mitotically and post-migration expressed in the inner nuclear layer exclusively within the amacrine interneuron lineage' The syntax could be better, for example 'Nr4a2a:eGFP is expressed exclusively in post-mitotic and post-migration interneurons within the amacrine cell lineage.'

'Using the Tg(nr4a2a:eGFP) line, we performed in vivo time-lapse imaging experiments to correlate the timing of transgene expression the phases of neural differentiation.' Do you mean 'throughout the phases of neural differentiation?'

'The density and distribution at this late time point is similar to that observed with antibody staining for dopaminergic neurons as shown later.' Not clear whether the similarity refers to in situ (4G) or eGFP (4N).

'Ptf1a is a transcription factor that is necessary and sufficient for the development of all amacrine cells (Jusuf et al., 2011), of which DA neurons are a subtype.'

It's ok to remind the reader about the transient expression of ptf1a in amacrine cells at this point, but the 'necessary and sufficient' and 'of which DA neurons are a subtype' sounds like getting too far ahead. This is likely material for the Discussion section. It seems likely that other transcription factors, like *atoh7*, might also be needed.

The brightness scale in Fig. 5 is a little dark, both on screen and in hardcopy. This is particularly the case for 5A-5C.

'As previously described, Ptf1a:RFP can be first detected around 35 hpf, when amacrine cells start to differentiate (Jusuf and Harris, 2009). At this time, the Nr4a2a:eGFP transgene is starting to be detected in the ganglion cell layer in Ptf1a unlabelled ganglion cells (Figure 5A).'

The reviewer doesn't see any Nr4a2a (green) cells in Fig. 5A (35 hpf). They are not visible in hardcopy, and barely visible on monitor at 37 hpf (Fig. 5B). In Fig. 5I the upper left dot is seen to reside inside a barely green Nr4a2a cell. Can the contrast and brightness be improved?

'Ptf1a:RFP labeled horizontal cells that start migrating back apically around 45 hpf never express Nr4a2a:eGFP (Figure 5F - H).'

In Fig. 5H 'HC' appears twice, once in magenta, and below it, in yellow. The reviewer didn't understand the yellow 'HC' label, as the text indicates HC's only express the Ptf1a (magenta) marker. Perhaps the yellow text should be removed. Otherwise this is a beautiful point.

Fig. 6 legend:

'A, B Expression of transgenic Nr4a2a:eGFP consistently labels cells in the amacrine layer (ACL) and additionally cells in the ganglion cell layer (GCL) as seen in 5 dpf retinal sections. C - H Even though rarely observed in retinal sections at 5 dpf, nr4a2a in situ labelled cells (purple) can be observed in this layer at earlier 48 hpf or 72 hpf in wholemount (C - E)' The phrase 'observed in this layer' is ambiguous. Replace with 'observed in the GC layer'.

'Atoh7:RFP is first detected at 28 hpf, just prior to terminal mitosis of the first born ganglion cells first start differentiating (Poggi et al., 2005).' Fix the sentence.

'Tyrosine hydroxylase immunoreactive cells co-labelled with our eGFP transgene in the retina (100%, n = 15 embryos),' In other sections the authors provide the number of cells that co-localized. The reviewer didn't understand the switch from co-labelled cells to co-labelled embryos, or what a co-labelled embryo might mean. Is this an embryo with an example of a co-labeled cell in one or both retinas? This counting method needs definition. It's a common issue.

Animals/retinas/cells are three different indexes. The reviewer prefers cells for statistics, of course bearing in mind that all the cells shouldn't be in 1 retina.

Results section: 'Nr4a2a:eGFP expression in distinct non-dopaminergic retinal amacrine cells'

'A common observation across mammalian retina is that Nr4a2 is expressed in many more amacrine retinal neurons that are not labelled by dopaminergic markers, such as tyrosine

hydroxylase. In mammals these have been categorised as GABAergic, suggesting that Nr4a2 may be a particular marker of this large subpopulation of amacrine cells.'

JCN is conservative in style and recommends reserving the Results section for results, and the Discussion section for discussion. These sentences sound like Discussion. The reviewer thinks a short contextual sentence in the Results is ok, but it would in any case need a reference.

Fig. 9 legend 'A Some, but not all GABAergic amacrine interneuron subpopulations were co-labelled with cells expressing varying degrees of Nr4a2a:eGFP (white dots).' For Fig. 9A, please define the 'stars', presumably, GABA, but no co-labeled eGFP.

Fig. 10 is a powerful summary. Once again, 'n' is the number of embryos. This should be defined in the legend. The reviewer didn't particularly like embryos as the value for 'n'. Why not the number of co-labelled cells? Probably both should be given.

Discussion:

'Here, we present a novel transgenic line using the promoter of the Nr4a2a gene that is expressed very early in neuron development and can be used in conjunction to the specific DA lines to visualise DA neurons from the very beginning. This will be useful for genetic studies, which affect early development and/or differentiation of neurons at stages at which they do not yet express the final DA machinery, including for example the dopamine transporter and tyrosine hydroxylase.'

The reviewer likes the author's developmental theory, but notes that tyrosine hydroxylase antigenicity is only shown at 5 dpf. Though TH may well be the last gene activated in the sequence, the paper does not present a time sequence for TH expression to prove that point.

'suggesting that at least most of these cells can be labelled until this age. we are interested in' Fix the punctuation.

Discussion Section: 'Developmental lineage specific expression of Tg(nr4a2a:eGFP) in the retina'

'Unlike the very transient nature of the nr4a2a mRNA expression, our transgenic line enables us to study the role of resulting differentiation of all neurons that express Nr4a2a.'

Ok, but you have repeated this point several times. The advocacy for the transgenic line deserves its own discussion paragraph. Then the point would not need constant repetition.

Second sentence:

'The nr4a2a RNA signal and Nr4a2a:eGFP signals detected within the retina after amacrine cells have started differentiation (Jusuf and Harris, 2009) exclusively in the INL and GCL, and our time-lapse imaging confirm, that these cells have exited the cell cycle and no longer undergo apical cell divisions as well as having migrated to their future laminar location.'

This sentence also could use some sorting out. The identity of nr4a2a:GFP cells in the GCL is not really a topic of this manuscript. Very few of them are immuno-reactive for amacrine cell markers. The authors earlier concluded that these are ganglion cells, that is, that not all nr4a2a:GFP cells in the GCL are amacrine cells.

'In contrast to previous zebrafish retinal nr4a2a expression data, a transgenic zebrafish line labelling differentiated DA neurons by driving reporter GFP under the dopamine transporter promoter also expressed in the GCL, though these might be displaced amacrine cells (see Figure 4B, 7A Xi et al., 2011).'

The reviewer did not find any GFP labeled GC's or displaced amacrine cells in Fig. 7A of Xi et al, 2011.

'We believe that transgenically labelled cells within the GCL faithfully recapitulate nr4a2a promoter activation, with our in situ expression indicating that Nr4a2a is expressed, though extremely shortly in this layer.' Use 'transiently' instead of 'shortly' But more important, the sentence does not belong in this paragraph, which otherwise compares mouse to zebrafish.

'Ganglion and amacrine cell start differentiation in the zebrafish retina at 28 hpf and 35 hpf,' 'cells start differentiation'

Reviewer: 3

Comments for the Authors
Goodings et al.

This manuscript concerns the expression of the nuclear receptor subfamily 4 group A member 2 (Nr4a2) in the zebra fish with a primary focus on its expression in the retina, as well as some comments on its expression in the nervous system. Nr4a2 expression was mapped by developing a Nr4a2 zebrafish transgenic and by in situ hybridization. Nr4a2 expression in dopaminergic amacrine cells and other amacrine cells was confirmed using immunohistochemistry. In addition, studies showed that Nr4a2 expression occurs in specific progenitor lineage that gives rise to amacrine and ganglion cells.

The development of the zebrafish line and most of the basic findings of this study are well documented, although there are some concerns about the characterization of the amacrine cells. This concern has both theoretical and practical components that will need to be addressed (see below). The text is well written, and most figures adequately illustrate the findings of this work.

Major concerns / issues:

1) Nr4a2 expression illustrated in figure 1 differs markedly when comparing the transgenic and in situ patterns, as noted by the authors. The transgenic expression is greater and clearly includes more than cells in the dopaminergic nuclei.

- Figure 1 would suggest mis-expression of Nr4a2 in the transgenic model or alternatively Nr4a2 is transiently expressed in numerous non-dopaminergic cells along the entire neuraxis. The authors should further elaborate on this concern / issue in the Discussion.

Nr4a2 expression in non-dopaminergic cells along the neuraxis is consistent with the findings in the retina (figures 8 and 9). The authors should test if GABA immunoreactive cells in the central nervous system express Nr4a2.

2) There is an issue concerning the cellular expression of Nr4a2 in the retina, and if it is expressed by a sub-group of GABAergic amacrine cells of which the dopaminergic cells is a member or if it is expressed in GABAergic cells and separately in dopaminergic cells.

In mammals, in all cases that I know of, dopaminergic cells also express GABA immunoreactivity. If this is true in zebrafish, then might a better interpretation of these findings is that Nr4a2 is expressed in a sub-group of GABAergic amacrine cells?

- The authors should determine if zebrafish tyrosine hydroxylase immunoreactive (dopaminergic) cells express GABA and other GABAergic markers, or point to the literature where this is properly documented. This experiment would help sort out if dopaminergic amacrine cells are a subgroup of GABAergic amacrine cells in the zebra fish retina.

Results; Glycine antibodies should be used as a negative control to test if Nr4a2 expression is only in GABA immunoreactive amacrine cells.

3) An additional concern is that the antibodies chosen to identify subgroups of GABAergic amacrine cells in the zebra fish may or may not mark GABAergic amacrine cells in this species. The authors need to either quote high quality studies of the zebra fish retina that show that the antibodies they

are using are indeed markers of GABAergic amacrine cells in zebra fish or they need to do the control studies. As is, it is difficult to evaluate the findings illustrated in figures 8 and 9, and the authors' assertions regarding Nr4a2 expression in specific GABAergic amacrine cell subgroups.

The authors should be aware that relying on the mammalian literature is problematic; for instance, the calcium binding proteins they are using are also expressed by ganglion cells in several mammalian species, and in horizontal cells. In some cases they are poorly expressed in amacrine cells.

Results; Antibody characterizations (pages 6-8): The authors should quote those papers that have used these particular antibodies in studies of the zebra fish retina and secondly in studies of mammalian retina. As is, the descriptions are inadequate to judge antibody specificity and the immunostaining patterns they produce in the zebra fish retina.

4) What accounts for the weak (figure 3A') and the strong (figure 6A, B; figure 8A,B,C) GFP labeling illustrated in the IPL?

If the labeling is due to dopaminergic cell processes, how does this pattern compare to a zebrafish retina immunostained with antibodies tyrosine hydroxylase? This should be illustrated to aid in the evaluation of the labeling pattern in the transgenic model.

If the labeling is due to dopaminergic and GABAergic cell processes, how does this pattern compare to a zebrafish retina immunostained with antibodies tyrosine hydroxylase and GABA? The type of experiment illustrated in figure 9A is to low a magnification to sort out this issue.

5) What accounts for the weak (figure 6A, B; figure 8A''', B''', C''') GFP labeling of cell bodies in the GCL?

The authors should determine if there is weak Nr4a2 expression in displaced amacrine cells and/or ganglion cells, and specifically test if GABA immunoreactive cells in the GCL weakly express GFP.

Results (figure 9): Why is there a lack of weak GFP cells in the GCL in all figures except 9H?

6) Figures: The figures need additional labeling. The major landmarks in figure 1 and 2 should be indicated. The retina figures should indicated the INL, IPL and GCL.

Moderate concerns:

1) There is an emphasis on Nr4a2 and its expression in dopaminergic neurons in this report, although beginning with the title and abstract the authors note that Nr4a2 is expressed in non-dopaminergic cells. This, I presume is because of Nr4a2 expression in non-dopamine immunoreactive, GABA immunoreactive cells in the retina (Figures 8 and 9) and its expression outside of dopamine cell rich areas (Figures 1 and 2).

After having read this manuscript, I was not sure if Nr4a2 is mainly expressed in dopamine containing cells, or if it has a wider expression in the nervous system, and should be more properly be considered a marker of a subgroup of GABA immunoreactive cells (which may include dopaminergic cells). The authors should consider the clarity of the message they are trying to send regarding the cellular labeling pattern of Nr4a2 shown in this report, and if needed edit the text appropriately.

2) Scholarship; the literature citations, especially those concerning dopaminergic amacrine cells, and the function of dopamine in the retina is dated, and should be revised to include key modern references. Of particular note is the first paragraph of the Introduction and parts of the Discussion.

3) Introduction (page 4, line 22): The statement that Nr4a2 expression is in distinct amacrine cell types is an over statement, and needs to be revised. At best, this study shows Nr4a2 expression in some GABAergic amacrine cells (that are not neurochemically defined) and the entire dopaminergic amacrine cell population. See figures 7- 9.

4) Methods (page 6, line 10) / Results: There is a concern regarding excessive fixation of the retina for the immunohistochemical studies. Many antibodies are fixation sensitive and the weak / no immunostaining seen for some of the antibodies could be due to tissue preparation. The authors should consider, if they need to repeat their experiments using shorter fixation times.

Methods (page 10) / Results and figure 10: Related is a concern regarding the measurement of immunostaining intensity. The text could use a more detailed explanation of the details of this experiment, including internal controls for variability of antibodies, tissue section thicknesses, tissue fixation, how cells were selected or rejected from analysis, if cells were selected randomly (and if so on what basis) for analysis, how were partially sectioned cell bodies recognized, and how the values were compared across the 6 embryos.

5) Results and figure 9: The authors should document the percentage of labeling in the experiments using the different cell markers. Include - number of cells counted, number of cells expressing GFP, number of cells expressing the antibody immunoreactivity.

Minor concerns & issues:

Abstract (line 11): some indication of the percentage of labeled amacrine cells should be given.

Abstract (line 17): need to be revised to give a better sense of the number of labeled / co-labeled cells.

Introduction (page 4, lines 10-13): This sentence suggests Nr4a2 expression in both GABAergic and dopaminergic cells in the retina; as noted above, this is a confusing statement and it should be revised.

Results (page 12, line 18): "most cells" is vague - Please provide the number of cells labeled, co-labeled and how many retinas were studied.

AUTHORS' RESPONSE LETTER:

12 January 2017

Dear Dr. Finger and Dr. Hof,

Thank you for the review and invitation to submit a re-review for our manuscript "In vivo expression of Nurr1/Nr4a2a in developing retinal amacrine subtypes in zebrafish Tg(nr4a2a:eGFP) transgenics" (JCN-16-0184) in the Journal of Comparative Neurology.

In this work, we describe the developmental expression of the Nr4a2 transcription factor in the retina, assessing its expression timing, lineage and correlation to different subtypes of neurons.

Nr4a2 has been implicated in the development of dopaminergic and possibly other GABAergic neurons across the CNS of vertebrates, and this work highlights the utility of our newly generated Nr4a2a:eGFP zebrafish transgenic line for better understanding its role during neurogenesis.

We thank both editors and three reviewers for their comments and suggestions, which we have addressed to improve the manuscript.

We addressed all of the issues raised and have copied in the original reviewers' comments below, followed by a point by point description of relevant revisions as well as tracking all changes in the manuscript document. We also attach this response as a file, as we have integrated some figures to show the additional experimentation where relevant for the benefit of the editors and reviewers.

Here we list the main revisions performed:

- 1) We have rewritten the antibody methods section to cite evidence for each antibody's specificity for zebrafish.
- 2) The figures have been reworked particularly to increase brightness and add labelling.
- 3) Additionally to fixing all noted errors and clarifying various sentences, a native English speaker has proof-read the entire manuscript.
- 4) We added the numbers of cells and the number of embryos analysed for all results.
- 5) Additional experiments were performed to address some of the questions. Some sample images are provided in this letter and where we felt relevant, the additional data was integrated into the manuscript.
- 6) More detail was added to the methods analysis section as requested.
- 7) The discussion was substantially reworked to address a number of reviewers' comments.

Regarding color figures, following figures can be reproduced in color online and in black and white on print:

Figures 1, 2, 4, 6, 10.

Kind regards,

Patricia Jusuf

POINT BY POINT RESPONSE - WE HAVE ALSO UPLOADED THIS RESPONSE LETTER, WHICH HAS SOME IMAGES INSERTED AND ALSO USES BLUE FONT TO MORE EASILY DISTINGUISH THE RESPONSES FROM EACH QUESTION.

Reviewer: 1

Comments for the Authors

This paper describes a solid investigation of the expression of Nr4a2a in the zebrafish retina. It is a carefully done set of experiments and is well interpreted and put into context of previous results. I have some minor concerns.

1. I am sure it is much easier to appreciate the co-labelling with various markers under the microscope than on the page or computer screen. I think it is possible, but a struggle, to see some of the points the authors are making on the figures I looked at. I would advise the authors to brighten up the figures and to do even larger enlargements of some of the key findings so that readers do not have to squint to see things.

The brightness and contrast of all figures have been adjusted.

2. The grammar is a little loose in places. Some careful editorial work or a good grammar checker is essential.

A native English speaker colleague has helped us to remedy grammatical errors and edit the manuscript.

Reviewer: 2

Comments for the Authors

Overview:

Amacrine cells are retinal interneurons that lie at the border between the inner nuclear layer and the inner plexiform layer. As many as 30 different dendritic branching patterns distinguish amacrine cell subtypes. In the late 70's and early 80's, with the development of immunohistochemistry, many of these subtypes were found to have unique immunogenicity, with some reactive for substance P, others for parvalbumin, and so forth. Morphological classification was confirmed by selective immunoreactivity, and in many cases, these patterns could be seen across species, confirming not only the legitimacy of the morphological subtypes, but also the cross-species specificity of antibodies. The manuscript of Goodings et al examines the origins of these different antigenic types during retinal development in zebrafish. They test the important idea that development of amacrine subtypes follows a time sequence in activation of transcription factors. In particular, they follow the tyrosine hydroxylase immunoreactive subtype, which follows the sequence Atoh7=>PTf1a=>Nr4a2a=>tyrosine hydroxylase. Among cells following this sequence, tyrosine hydroxylase is the only immunoreactive peptide found in a screen of 8 peptides known to be selective for amacrine types. To be sure, not all amacrine cells following the sequence Atoh7=>Ptfa=>Nr4a2a produced tyrosine hydroxylase. About half of these were simply 'GABAergic'. Perhaps further transcription factors in the sequence leading to tyrosine hydroxylase immunoreactivity are yet to be found. The intellectual content of this research line is very exciting.

Since this is an antibody

paper, the reviewer needs to consider the application of the Clifford Saper JCN editorial 'An Open Letter to Our Readers on the Use of Antibodies'. This elegant editorial details the stringency with which one may be certain that an antibody recognizes an epitope, with the highest certainty being that mutants for the epitope are unreactive, but pointing out that even in this case, one can't be certain that the antibody wasn't reactive with a downstream product. The current study uses antibodies characterized in rodents to label zebrafish. The issue needs to be addressed. Though the Saper editorial does not mention this situation, the reviewer thinks that a characterized antibody

that labels an amacrine subtype in both rodents and zebrafish is a reasonably stringent criterion for antibody specificity in the uncharacterized species.

We have addressed the zebrafish specificity of the relevant antibodies as shown in the completely revised antibody section in the methods. We have not copied the entire paragraph into this letter again.

We performed the Western blot for parvalbumin in whole zebrafish homogenate as shown in following image. We have referred to this as “data not shown”, but could include as Supplementary figure, if required.

Nonetheless, for tyrosine hydroxylase, there are two paralogues th1 and th2 in zebrafish. Perhaps the authors should consider a test of antibody reactivity for tyrosine hydroxylase in a morpholino knockout of th1. In any case the TH paralogue issue should be addressed.

In the relevant methods section under TH specificity for zebrafish we cite references that directly answer this query:

“This commercial antibody was raised against TH purified from rat PC12 cells and recognises an epitope outside of the regulatory N-terminus and a 62 kDa band in immunoblots of zebrafish brain (Uyttebroek et al., 2010). In zebrafish CNS this antibody exclusively labels TH1 as shown by colocalisation with th1, but not th2 in situ hybridisation (Chen et al., 2009). Additionally, immunolabelling with this antibody is abolished following morpholino mediated knockdown specifically of th1 in embryonic zebrafish CNS (Kuscha et al., 2012).

The Saper editorial also does not deal with criteria for credibility of transgenic reporter lines. One of the achievements of this manuscript is a transgenic reporter line for Nr4a2a. The authors have compared the reporter eGFP fluorescence to the developmental sequence of in situ hybridization for Nr4a2a mRNA. This was carefully done, and the transgene reporter line is credible.

While the thematic material of the manuscript is important, the text is plagued by syntax problems, some of which make idea structure difficult to parse. Journals don't supply copy editors any more. The authors will need to find their own. The 'Conclusions' section was strange, reading more like a prospectus than the product of experimentation.

We directly address this by recruited the assistance of English native speakers to proof-read the manuscript. All changes are tracked using the reviewer function in word.

Specific comments:

The authors use the words 'perdure, perdurant, and perdurance' 13 times in manuscript and legend. Presumably these refer to the 'half-life of eGFP'. While the word is very cool, it's extensive use tends to beg the question, what is the eGFP half-life? Are adult retinas fluorescent in the transgenic, and if so, in which cells and regions? That is the problem with 'perdure' so perdurantly. Otherwise it is completely reasonable to expect that eGFP need not overlap either the time or subcellular localization of the native Nr4a2a protein.

The half-life of eGFP is ~26 hours. We added following extension to the sentence:

“The transgenic line shows strong eGFP expression throughout the CNS and is maintained in regions that have now started downregulating endogenous expression (white arrowheads, Figure 1), due to the perdurance of the eGFP transgene with half-life of > 1 day (Corish and Tyler-Smith 1999, Andersen... Molin 1998) (Figure 1E –).”

We also deleted some of these terms to reduce repetition.

At 14 dpf zebrafish larvae, we find GFP positive cells in the same retinal layers (i.e. similar populations), with a relatively reduced number, though we have not done a thorough classification of these at these stages, which we believe may be outside the scope of this comment.

Abstract:

‘The context of Nr4a2 expression (which cells when within which gene networks) and function remains poorly understood.’ Fix the sentence.

This sentence has been replaced by: “Little is known about which cells express Nr4a2 at which developmental stage. Furthermore, whether Nr4a2 functions in combination with other genes is poorly understood.”

‘Nr4a2a:eGFP is confined to a specific progenitor lineage identified by sequential expression of atonal bhlh transcription factor 7 and pancreas transcription factor 1 a, which primarily drive ganglion and amacrine fates respectively.’ This MS shows atoh7 followed by PTF1a drives an amacrine cell fate. Ganglion cell fates are less well defined, but start with atoh7, and probably sometimes followed by Nr4a2a, as seen in the MS. The sentence is unclear because it tries to do too much.

This sentence has been shortened:

“Nr4a2a:eGFP is confined to a specific progenitor lineage identified by sequential expression of atonal bhlh transcription factor 7 and pancreas transcription factor 1 a.”

Methods

‘Wholemounds embryos were dehydrated serially’ ‘wholemount embryos’

The “s” has been deleted.

Results

‘A detailed temporal time series were conducted to follow the onset and perdurance of the eGFP signal’ ‘was conducted’

This has been corrected.

‘The rabbit anti-secretagogin antibody (RRID: AB_203 4060) used shows identical staining to a commercial and non-commercial secretagogin antibody’ It sounds like the authors tried 3 secretagogin antibodies, alternatively they are quoting results obtained elsewhere. Please elaborate.

We have two commercial antibodies with identical labeling in zebrafish (see image supplied here). Note as sometime occurs, the tissue also has background staining, but the actual cells labeled (asterisks) show 100% green – red co-localisation.

The third secretagogin one shows identical staining in mammals and has been shown to be specific by Western blot.

This section has been clarified and now states:

“The rabbit anti-secretagogin antibody (RRID: AB_2034060) used shows identical staining in zebrafish to a commercial goat anti-secretagogin antibody not used for analysis in this study (data not shown). In mammalian species both of these antibodies label the same cells as a non-commercial secretagogin antibody that recognise the 32 kDa predicted band in mouse retina and cerebellum (Puthussery et al., 2010; Weltzien et al., 2014).”

In Fig. 1, as in Fig. 2, the authors might want to include the column headers ‘dorsal’ and ‘lateral’.

These headers have been added.

Results header: ‘Nr4a2a:eGFP is expressed post-mitotically and post-migration expressed in the inner nuclear layer exclusively within the amacrine interneuron lineage’ The syntax could be better, for example ‘Nr4a2a:eGFP is expressed exclusively in post-mitotic and post-migration interneurons within the amacrine cell lineage.’

This heading has been revised to:

“In the inner nuclear layer Nr4a2a:eGFP is expressed exclusively in post-mitotic and post-migratory amacrine interneurons”

‘Using the Tg(nr4a2a:eGFP) line, we performed in vivo time-lapse imaging experiments to correlate the timing of transgene expression the phases of neural differentiation.’ Do you mean ‘throughout the phases of neural differentiation?’

We correlate the timing not only throughout, but also TO each phase, i.e. is its expression correlated with a particular phase?

We have rephrased this sentence:

“Using the Tg(nr4a2a:eGFP) line, we performed in vivo time-lapse imaging experiments to correlate the timing of transgene expression to the different stages of neural differentiation.

‘The density and distribution at this late time point is similar to that observed with antibody staining for dopaminergic neurons as shown later.’ Not clear whether the similarity refers to in situ (4G) or eGFP (4N).

This has been clarified:

“Using the Tg(nr4a2a:eGFP) line, we performed in vivo time-lapse imaging experiments to correlate the timing of transgene expression to the different stages of neural differentiation.”

‘Ptf1a is a transcription factor that is necessary and sufficient for the development of all amacrine cells (Jusuf et al., 2011), of which DA neurons are a subtype.’

It’s ok to remind the reader about the transient expression of ptf1a in amacrine cells at this point, but the ‘necessary and sufficient’ and ‘of which DA neurons are a subtype’ sounds like getting too far ahead. This is likely material for the Discussion section. It seems likely that other transcription factors, like atoh7, might also be needed.

This sentence provides published background information and simply states that Ptf1a is necessary and sufficient for amacrine cells as we previously showed in Jusuf et al., 2011 and that DA in the retina are amacrine cells. The sentence does not discuss anything about Ptf1a being sufficient for DA neurons, as we believe it might have been read by the reviewer.

We have rephrased to now state:

“Ptf1a is a transcription factor that is expressed transiently during the development of all amacrine cells (Jusuf et al., 2011), of which DA neurons are a subtype.”

The brightness scale in Fig. 5 is a little dark, both on screen and in hardcopy. This is particularly the case for 5A-5C.

‘As previously described, Ptf1a:RFP can be first detected around 35 hpf, when amacrine cells start to differentiate (Jusuf and Harris, 2009). At this time, the Nr4a2a:eGFP transgene is starting to be detected in the ganglion cell layer in Ptf1a unlabelled ganglion cells (Figure 5A).’

The reviewer doesn't see any Nr4a2a (green) cells in Fig. 5A (35 hpf). They are not visible in hardcopy, and barely visible on monitor at 37 hpf (Fig. 5B). In Fig. 5I the upper left dot is seen to reside inside a barely green Nr4a2a cell. Can the contrast and brightness be improved?

The brightness of the entire figure was increased a little bit. However, we believe it is important for correct representation of the data not to change the brightness of individual panels. As is, the panels can be directly compared with each other. Increasing the brightness any further makes the later time points too over-saturated.

We updated the results sentence to state:

“At this time, the Nr4a2a:eGFP transgene is just starting to be detected faintly in the ganglion cell layer in Ptf1a unlabelled ganglion cells (Figure 5A).”

A sentence was added to the figure legend:

“The brightness of the entire figure is adjusted simultaneously, such that individual panels are directly comparable.”

‘Ptf1a:RFP labeled horizontal cells that start migrating back apically around 45 hpf never express Nr4a2a:eGFP (Figure 5F - H).’ In Fig. 5H ‘HC’ appears twice, once in magenta, and below it, in yellow. The reviewer didn't understand the yellow ‘HC’ label, as the text indicates HC's only express the Ptf1a (magenta) marker. Perhaps the yellow text should be removed. Otherwise this is a beautiful point.

Apologies, one of the two “HC” labels is indeed meant to say “AC” and has been updated in the figure.

Fig. 6 legend:

‘A, B Expression of transgenic Nr4a2a:eGFP consistently labels cells in the amacrine layer (ACL) and additionally cells in the ganglion cell layer (GCL) as seen in 5 dpf retinal sections. C - H Even though rarely observed in retinal sections at 5 dpf, nr4a2a in situ labelled cells (purple) can be observed in this layer at earlier 48 hpf or 72 hpf in wholemount (C - E)’ The phrase ‘observed in this layer’ is ambiguous. Replace with ‘observed in the GC layer’.

We have replaced as suggested.

‘Atoh7:RFP is first detected at 28 hpf, just prior to terminal mitosis of the first born ganglion cells first start differentiating (Poggi et al., 2005).’ Fix the sentence.

We added a “the” to now state “prior to THE terminal mitosis”.

‘Tyrosine hydroxylase immunoreactive cells co-labelled with our eGFP transgene in the retina (100%, n = 15 embryos),’ In other sections the authors provide the number of cells that co-localized. The reviewer didn’t understand the switch from co-labelled cells to co-labelled embryos, or what a co-labelled embryo might mean. Is this an embryo with an example of a co-labeled cell in one or both retinas? This counting method needs definition. It’s a common issue. Animals/retinas/cells are three different indexes. The reviewer prefers cells for statistics, of course bearing in mind that all the cells shouldn’t be in 1 retina.

The cell number has now been added to each relevant results section (not copied into this rebuttal letter, as there were many additions that are highlighted with track changes). The methods section also has a relevant sentence added: “The number of cells analysed for each are indicated in the results section.”

The embryo number indeed only shows that the cells are not all analysed in 1 retina, but were not meant to be read as “co-labelled embryo”, which of course makes little sense. We hope addition of the cell numbers have also clarified what the number of embryo indicates.

Results section: ‘Nr4a2a:eGFP expression in distinct non-dopaminergic retinal amacrine cells’
‘A common observation across mammalian retina is that Nr4a2 is expressed in many more amacrine retinal neurons that are not labelled by dopaminergic markers, such as tyrosine hydroxylase. In mammals these have been categorised as GABAergic, suggesting that Nr4a2 may be a particular marker of this large subpopulation of amacrine cells.’

JCN is conservative in style and recommends reserving the Results section for results, and the Discussion section for discussion. These sentences sound like Discussion. The reviewer thinks a short contextual sentence in the Results is ok, but it would in any case need a reference.

This has been revised and the second sentence as stated removed from this results section.

Fig. 9 legend ‘A Some, but not all GABAergic amacrine interneuron subpopulations were co-labelled with cells expressing varying degrees of Nr4a2a:eGFP (white dots).’ For Fig. 9A, please define the ‘stars’, presumably, GABA, but no co-labeled eGFP.

This has been revised:

“A Some of the GABAergic amacrine interneuron subpopulations were co-labelled with cells expressing varying degrees of Nr4a2a:eGFP (white dots), though GABAergic amacrine cells that did not express any Nr4a2a:eGFP were also observed (white asterisks).”

Fig. 10 is a powerful summary. Once again, ‘n’ is the number of embryos. This should be defined in the legend. The reviewer didn’t particularly like embryos as the value for ‘n’. Why not the number of co-labelled cells? Probably both should be given.

This has now been added to each relevant results section (not copied into this rebuttal letter, as there were many additions that are highlighted with track changes). The methods section also has a relevant sentence added: “The number of cells analysed for each are indicated in the relevant section.”

We also clarified in this method section that the counts are done only in relation to the immunolabel (how many of the immunolabelled populations are expressing Nr4a2a:eGFP), but not vice versa, except for GABA, where we saw that Nr4a2 are a complete subpopulation of GABAergic cells.

The figure has been changed to show the number of cells and the figure legend now states:
“N = number of cells analysed for each immunohistochemical marker.”
The relevant results section has been expanded to show the number of cells and embryos.

Discussion:

‘Here, we present a novel transgenic line using the promoter of the Nr4a2a gene that is expressed very early in neuron development and can be used in conjunction to the specific DA lines to visualise DA neurons from the very beginning. This will be useful for genetic studies, which affect early development and/or differentiation of neurons at stages at which they do not yet express the final DA machinery, including for example the dopamine transporter and tyrosine hydroxylase.’
The reviewer likes the author’s developmental theory, but notes that tyrosine hydroxylase antigenicity is only shown at 5 dpf. Though TH may well be the last gene activated in the sequence, the paper does not present a time sequence for TH expression to prove that point.

Here, we simply state that in time-lapse for example, cells identified with dopaminergic specific markers at later time-points (e.g. transgenes for TH) will also be eGFP positive in this line. By analyzing such movies in reverse, these green cells can be tracked to earlier developmental time points, even when the dopaminergic marker has not yet been expressed.

This is not a unique feature of our line, as a fluorescently marker tagged histone nuclear label could also be used similar, but such marker often results in labeling so many cells, that they are more difficult to distinguish and track in 3D and time.

We added a sentence to clarify this in the discussion.

“This allows us to perform time-lapse experiments, where cells that are identified to be dopaminergic by late differentiation markers at later time-points can then be tracked back in time and followed by expression of the eGFP in this transgenic line.”

‘suggesting that at least most of these cells can be labelled until this age. we are interested in’ Fix the punctuation.

This has been fixed:

“suggesting that at least most of these cells can be labelled until this age. Thus, the Nr4a2a:eGFP reporter labels...”

Discussion Section: ‘Developmental lineage specific expression of Tg(nr4a2a:eGFP) in the retina’

‘Unlike the very transient nature of the nr4a2a mRNA expression, our transgenic line enables us to study the role of resulting differentiation of all neurons that express Nr4a2a.’

Ok, but you have repeated this point several times. The advocacy for the transgenic line deserves its own discussion paragraph. Then the point would not need constant repetition.

We have made a separate heading, shortened the relevant discussion of this issue, and deleted subsequent repetitions.

Second sentence:

‘The nr4a2a RNA signal and Nr4a2a:eGFP signals detected within the retina after amacrine cells have started differentiation (Jusuf and Harris, 2009) exclusively in the INL and GCL, and our time-

lapse imaging confirm, that these cells have exited the cell cycle and no longer undergo apical cell divisions as well as having migrated to their future laminar location.'

This sentence also could use some sorting out. The identity of nr4a2a:GFP cells in the GCL is not really a topic of this manuscript. Very few of them are immuno-reactive for amacrine cell markers. The authors earlier concluded that these are ganglion cells, that is, that not all nr4a2a:GFP cells in the GCL are amacrine cells.

We have revised this sentence to state:

"The expression of nr4a2a RNA and Nr4a2a:eGFP in the INL is detected after amacrine cell differentiation has begun (Jusuf and Harris, 2009). The time-lapse imaging confirms, that these cells have exited the cell cycle and completed migration to the amacrine layer.

'In contrast to previous zebrafish retinal nr4a2a expression data, a transgenic zebrafish line labelling differentiated DA neurons by driving reporter GFP under the dopamine transporter promoter also expressed in the GCL, though these might be displaced amacrine cells (see Figure 4B, 7A Xi et al., 2011).'

The reviewer did not find any GFP labeled GC's or displaced amacrine cells in Fig. 7A of Xi et al, 2011.

In this figure (Fig. 7A of Xi et al., 2011) in the eye shown on the left, there are 5 cells to the left of the IPL (some red diffuse background staining indicates the IPL layer), which is the ganglion cell layer.

'We believe that transgenically labelled cells within the GCL faithfully recapitulate nr4a2a promoter activation, with our in situ expression indicating that Nr4a2a is expressed, though extremely shortly in this layer.' Use 'transiently' instead of 'shortly' But more important, the sentence does not belong in this paragraph, which otherwise compares mouse to zebrafish.

We respectfully disagree. The entire paragraph centres around the positive labeled cells in the ganglion cell layer. This includes mentioning previous work in which similar observation is found in mouse, but the focus of the paragraph is not a zebrafish versus mouse comparison. Thus, this sentence directly summarises / concludes this paragraph.

'Ganglion and amacrine cell start differentiation in the zebrafish retina at 28 hpf and 35 hpf,' 'cells start differentiation'

This sentence has been corrected.

Reviewer: 3

Comments for the Authors
Goodings et al.

This manuscript concerns the expression of the nuclear receptor subfamily 4 group A member 2 (Nr4a2) in the zebra fish with a primary focus on its expression in the retina, as well as some comments on its expression in the nervous system. Nr4a2 expression was mapped by developing a Nr4a2 zebrafish transgenic and by in situ hybridization. Nr4a2 expression in dopaminergic amacrine cells and other amacrine cells was confirmed using immunohistochemistry. In addition, studies showed that Nr4a2 expression occurs in specific progenitor lineage that gives rise to amacrine and ganglion cells.

The development of the zebrafish line and most of the basic findings of this study are well documented, although there are some concerns about the characterization of the amacrine cells. This concern has both theoretical and practical components that will need to be addressed (see below). The text is well written, and most figures adequately illustrate the findings of this work.

Major concerns / issues:

1) Nr4a2 expression illustrated in figure 1 differs markedly when comparing the transgenic and in situ patterns, as noted by the authors. The transgenic expression is greater and clearly includes more than cells in the dopaminergic nuclei.

- Figure 1 would suggest mis-expression of Nr4a2 in the transgenic model or alternatively Nr4a2 is transiently expressed in numerous non-dopaminergic cells along the entire neuraxis. The authors should further elaborate on this concern / issue in the Discussion.

A key finding of this work which is consistent with previous studies of Nr4a2 is in fact that it is much more widely expressed than dopaminergic neurons and our work confirms this in the retina. We would not be surprised that this is the case in other CNS regions as well. We interpret this to be a real finding (i.e. Nr4a2 is truly expressed in non-dopaminergic neurons) rather than a mis-expression in the transgenic model.

Nonetheless, we have focused our characterization in the retina, and we have re-emphasised this in the discussion.

We have also added a few sentences to clarify that indeed we do not have the required data to rule out such mis-expression in other areas by adding following to the end of this discussion section: "While we find similar co-labelling of endogenous nr4a2a mRNA and the transgene as well as eGFP only labelled cells in other CNS regions, the remainder of the discussion focuses only on the retina. A thorough timecourse analysis of co-labelling for relevant CNS areas of interest should be conducted as required, to determine whether the transgene faithfully recapitulates Nr4a2 expression in these regions. "

Nr4a2 expression in non-dopaminergic cells along the neuraxis is consistent with the findings in the retina (figures 8 and 9). The authors should test if GABA immunoreactive cells in the central nervous system express Nr4a2.

We have performed the necessary staining (GABA in Nr4a2 transgenic) and found that most of the Nr4a2:eGFP labeled cells were indeed GABAergic in other CNS regions (see pictures showing different CNS regions with GABA in red and GFP in green). There were many GABAergic cells that are not eGFP labeled and there were a few GFP cells that did not seem to be GABAergic. However, we have not done the required extended comprehensive analysis systematically per CNS region in adequate numbers to state statistically relevant numbers.

We have added a sentence to the discussion:

"Nr4a2 seems to be expressed in a distinct subpopulation of amacrine interneurons in the retina (rather than randomly in a similar percentage of each subtype). This was strikingly exemplified by the lack of Nr4a2 for most of the subtype markers tested. Beside TH, Nr4a2 cells in the retina seem to be exclusively GABAergic, though not all GABAergic cells express Nr4a2. In other CNS regions, we also observed Nr4a2 transgene expression primarily within GABAergic cells, though there were many more GABAergic cells that did not express Nr4a2. Thus, there may be region specific subtype distributions of Nr4a2.

2) There is an issue concerning the cellular expression of Nr4a2 in the retina, and if it is expressed by a sub-group of GABAergic amacrine cells of which the dopaminergic cells is a member or if it is expressed in GABAergic cells and separately in dopaminergic cells.

In mammals, in all cases that I know of, dopaminergic cells also express GABA immunoreactivity. If this is true in zebrafish, than might a better interpretation of these findings is that Nr4a2 is expressed in a sub-group of GABAergic amacrine cells?

- The authors should determine if zebrafish tyrosine hydroxylase immunoreactive (dopaminergic) cells express GABA and other GABAergic markers, or point to the literature where this is properly documented. This experiment would help sort out if dopaminergic amacrine cells are a subgroup of GABAergic amacrine cells in the zebra fish retina.

Results; Glycine antibodies should be used as a negative control to test if Nr4a2 expression is only in GABA immunoreactive amacrine cells.

We have done this staining and indeed found that TH+ cells in the zebrafish retina are GABAergic. An example is shown here (TH in red, GABA in green) – white asterisks are shown just above each of the two cells in this image, as not to obstruct the actual view. We have spelt this out in the text and revised our wording throughout the manuscript to keep this message consistent.

Glycine and GABA show complementary expression patterns in the zebrafish retina (Marc, R.E. and Cameron, D. 2002. A Molecular phenotype atlas of the zebrafish retina. *J Neurocyt.*30:593-654.

3) An additional concern is that the antibodies chosen to identify subgroups of GABAergic amacrine cells in the zebra fish may or may not mark GABAergic amacrine cells in this species. The authors need to either quote high quality studies of the zebra fish retina that show that the antibodies they are using are indeed markers of GABAergic amacrine cells in zebra fish or they need to do the control studies. As is, it is difficult to evaluate the findings illustrated in figures 8 and 9, and the authors' assertions regarding Nr4a2 expression in specific GABAergic amacrine cell subgroups.

We have revised the methods antibody section to address this. The GABA paragraph states: "The rabbit anti-gamma-aminobutyric acid antibody (RRID: AB_477652) was used to label a large subpopulation of amacrine cells, which express the GABA neurotransmitter. The antibody specifically recognises GABA in a dot blot assay (manufacturer's datasheet, species not mentioned). The structure of GABA is identical in all vertebrate species. Within the zebrafish retina, the staining pattern obtained using this GABA antibody is comparable with that obtained using antibodies against GAD65 and GAD67, the glutamic acid decarboxylates, which are the enzymes that convert glutamate to GABA (Connaughton et al., 1999)."

The authors should be aware that relying on the mammalian literature is problematic; for instance, the calcium binding proteins they are using are also expressed by ganglion cells in several mammalian species, and in horizontal cells. In some cases they are poorly expressed in amacrine cells.

Results; Antibody characterizations (pages 6-8): The authors should quote those papers that have used these particular antibodies in studies of the zebra fish retina and secondly in studies of mammalian retina. As is, the descriptions are inadequate to judge antibody specificity and the immunostaining patterns they produce in the zebra fish retina.

As mentioned for a similar comment from reviewer 1, we have revised the entire antibody specificity section in our methods to specifically discuss zebrafish relevant information. The two

antibodies this study actually uses to recognize the protein are tyrosine hydroxylase and GABA. For the remainder of the markers, while we have also provided evidence for specificity, for our study and for how we are using these as differential subtype / subpopulation markers, the data does not rely on them recognizing the actual zebrafish equivalent of the mammalian protein they are raised against.

4) What accounts for the weak (figure 3A') and the strong (figure 6A, B; figure 8A,B,C) GFP labeling illustrated in the IPL?

The much weaker GFP labeling is due to Figure 3 showing tissue that has been processed for in situ hybridization. During this process, the GFP signal actually disappears and needs to be recovered with an anti-GFP immunohistochemistry, which we find in practice never as good as the original transgene or GFP immunohistochemistry after standard processing without in situ processing. The processes are definitely much weaker, but also the cell body staining is much weaker in these preparations.

If the labeling is due to dopaminergic cell processes, how does this pattern compare to a zebrafish retina immunostained with antibodies tyrosine hydroxylase? This should be illustrated to aid in the evaluation of the labeling pattern in the transgenic model.

If the labeling is due to dopaminergic and GABAergic cell processes, how does this pattern compare to a zebrafish retina immunostained with antibodies tyrosine hydroxylase and GABA? The type of experiment illustrated in figure 9A is to low a magnification to sort out this issue.

The strong GFP labeling in the IPL is in fact primarily due to non-DA amacrine cells and we would presume that these belong to amacrine cells whose cell bodies are also labeled with much stronger GFP intensity. However, we have not assessed this directly (which could be done for example by transplanting cells from the transgenic line into WT hosts until we have sufficient isolated cells and tracing the morphology of those for which the processes are most easily seen back to the cell body). Given that the GFP is cytoplasmic, we believe this interpretation to be the most likely.

Close up images of the IPL of Nr4a2:eGFP stained with TH+ shows no co-localisation between strongly GFP and TH+ labeled processes and does not add any data to the study in our opinion.

5) What accounts for the weak (figure 6A, B; figure 8A''', B''', C''') GFP labeling of cell bodies in the GCL?

The authors should determine if there is weak Nr4a2 expression in displaced amacrine cells and/or ganglion cells, and specifically test if GABA immunoreactive cells in the GCL weakly express GFP.

PV labels all displaced amacrine cells, as it shows 100% correspondence in our Ptf1a:GFP transgenic line.

PV label in the GCL shows very little co-localisation with Nr4a2:eGFP (only 4%) as stated in the results. We interpret this to mean that Nr4a2a:eGFP is expressed specifically in displaced amacrine cells, which we previously show to be GABAergic and never glycinergic (Jusuf & Harris, 2009).

GABA is expressed developmentally in the zebrafish ganglion cells in the retina (Sandell et al. 1994, The development of GABA immunoreactivity in the retina of zebrafish. JCN:345(4):596-601 and was therefore not used to test this.

Results (figure 9): Why is there a lack of weak GFP cells in the GCL in all figures except 9H?

For co-localisation such as these, all images are photographed with an apotome, which takes a thin optical slice much thinner than the actual section. Because sections are not always collected onto the slide completely parallel in the apotome mode, differences in focal level will show up much more pronounced.

As part of comments raised above, we have increased the brightness of the figure, which has also made GFP positive cells in the GCL more visible in all panels.

6) Figures: The figures need additional labeling. The major landmarks in figure 1 and 2 should be indicated. The retina figures should indicated the INL, IPL and GCL. Labelling has been added to figures (and described in figure legends).

Moderate concerns:

1) There is an emphasis on Nr4a2 and its expression in dopaminergic neurons in this report, although beginning with the title and abstract the authors note that Nr4a2 is expressed in non-dopaminergic cells. This, I presume is because of Nr4a2 expression in non-dopamine immunoreactive, GABA immunoreactive cells in the retina (Figures 8 and 9) and its expression outside of dopamine cell rich areas (Figures 1 and 2).

After having read this manuscript, I was not sure if Nr4a2 is mainly expressed in dopamine containing cells, or if it has a wider expression in the nervous system, and should be more properly be considered a marker of a subgroup of GABA immunoreactive cells (which may include dopaminergic cells). The authors should consider the clarity of the message they are trying to send regarding the cellular labeling pattern of Nr4a2 shown in this report, and if needed edit the text appropriately.

The reviewer correctly interprets our finding. The text is written to address the overwhelming background of literature labeling Nr4a2 as a dopaminergic gene and dopaminergic marker and describing that it should indeed be interpreted as a GABA subgroup marker.

We have read through the manuscript and made adjustments to the text where we felt we could clarify this further, but did not want to loose the link to the focus or significance of this transcription factor for which it has most commonly been described as.

2) Scholarship; the literature citations, especially those concerning dopaminergic amacrine cells, and the function of dopamine in the retina is dated, and should be revised to include key modern references. Of particular note is the first paragraph of the Introduction and parts of the Discussion.

We have addressed this concern and added relevant citations throughout the text.

3) Introduction (page 4, line 22): The statement that Nr4a2 expression is in distinct amacrine cell types is an over statement, and needs to be revised. At best, this study shows Nr4a2 expression in some GABAergic amacrine cells (that are not neurochemically defined) and the entire dopaminergic amacrine cell population. See figures 7- 9.

With “distinct”, we mean that it is not randomly expressed equally in all amacrine subtypes (e.g. 30% of GABA+, 30% in 5HT+, 30% in TH+, 30% in CB etc.). Our results very conclusively show that this is not the case. The fact that many of the subtype markers show very little co-localisation with Nr4a2 specifically showcases that Nr4a2 expression is limited to distinct subtypes.

4) Methods (page 6, line 10) / Results: There is a concern regarding excessive fixation of the retina for the immunohistochemical studies. Many antibodies are fixation sensitive and the weak / no immunostaining seen for some of the antibodies could be due to tissue preparation. The authors should consider, if they need to repeat their experiments using shorter fixation times.

Unless published in zebrafish before, all of the antibodies we use have been tested with various fixations including 2% PFA 1 hour, 3 hour, 4% PFA 1 hour, 3 hour primarily because immunostaining with antibodies made against mammalian proteins may not always work in zebrafish. Only CHAT was found to be extremely fixation sensitive and can be fixed for an absolute max. of 1 hour at 4%PFA. For GABA we in fact required glutaraldehyde in the fixation as well.

None of the antibodies showed “weak/no immunostaining”. All of them showed bright immunostaining, but not necessarily in Nr4a2a:eGFP expressing cells. Bright labeling in surrounding cells gives us confident that there are no technical issues with the fixation or antibody staining.

Methods (page 10) / Results and figure 10: Related is a concern regarding the measurement of immunostaining intensity. The text could use a more detailed explanation of the details of this experiment, including internal controls for variability of antibodies, tissue section thicknesses, tissue fixation, how cells were selected or rejected from analysis, if cells were selected randomly (and if so on what basis) for analysis, how were partially sectioned cell bodies recognized, and how the values were compared across the 6 embryos.

Tissue section thickness and fixation is constant.

Cells were chosen “randomly” with following criteria:

For immunohistochemical markers with relatively dense staining, central retinal sections were chosen near the section containing the optic nerve. This is purely, because these sections have the largest area and contain more cells.

For relatively rarely staining markers such as TH+ or 5HT+ or NY+, all labeled cells (up to 20 images per embryo) were analysed.

Sections that were folded over or had other processing artefacts (big tears) were excluded.

And each cell was considered one independent value in a binomial (colocalised or not?) way.

We have added some detail to the methods, but feel that other details such as constant tissue section thickness should be assumed standard.

5) Results and figure 9: The authors should document the percentage of labeling in the experiments using the different cell markers. Include - number of cells counted, number of cells expressing GFP, number of cells expressing the antibody immunoreactivity.

The quantification for this figure is provided in Figure 10 with the relevant number of cells and embryos. We have added the requested numbers to the main text in the results section as well.

Minor concerns & issues:

Abstract (line 11): some indication of the percentage of labeled amacrine cells should be given.

Abstract (line 17): need to be revised to give a better sense of the number of labeled / co-labeled cells.

We revised line 11 to address both of these issues:

"Nr4a2a:eGFP labelled multiple subtypes of neurons including xx% amacrine cells. These primarily co-labelled with tyrosine hydroxylase labelled dopaminergic amacrine cells, but mostly non-dopaminergic GABAergic amacrine populations."

Introduction (page 4, lines 10-13): This sentence suggests Nr4a2 expression in both GABAergic and dopaminergic cells in the retina; as noted above, this is confusing statement and it should be revised.

This has been revised to state:

"These studies suggest that in the retina, Nr4a2 may have a general role in the development of GABA-ergic including DAC."

Results (page 12, line 18): "most cells" is vague - Please provide the number of cells labeled, co-labeled and how many retinas were studies.

This has been added to the results section, which now states:

"In central retina, co-localisation with the eGFP transgene signal could be seen for 82% of cells also labelled with nr4a2a RNA in situ signal (n = 155 cells, asterisks in inserts, Figure 3a - a", B - B").). The immunohistochemical recovery of the GFP signal is variable after in situ hybridisation resulting in fewer and generally weaker GFP signal after such procedures (Figure 3). By 5 dpf, there were additional neurons positive only for Nr4a2a:eGFP in these regions."

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30 January 2017

Dear Dr. Finger and Dr. Hof,

Thank you for the final comments for our manuscript "***In vivo* expression of Nurr1/Nr4a2a in developing retinal amacrine subtypes in zebrafish Tg(nr4a2a:eGFP) transgenics**" (JCN-16-0184) in the Journal of Comparative Neurology.

We have attached a separate response letter outlining how we have addressed each of the remaining corrections (blue font used to highlight our responses).

We also attach the word document with all tracked changes as well as a "clean text" version with all of those changes accepted.

Kind regards,

Patricia Jusuf

Accepted

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ORIGINAL DECISION LETTER AND POINT BY POINT RESPONSE (IN BLUE FONT)

Dear Dr. Jusuf,

Your manuscript, JCN-16-0184.R1 In vivo expression of Nurr1 / Nr4a2a in developing retinal amacrine subtypes in zebrafish Tg(nr4a2a:eGFP) transgenics, has now been reviewed by our scientific referees and the comments are enclosed.

We are pleased to provisionally accept this work for publication pending the few minor changes listed by the reviewer and in our comments below. We appreciate your taking the time and your efforts to revise carefully your original work. Only a final review by the Editor-in-Chief will be required at this stage.

In your description of anti-secretagogin antibody you state that it "shows identical staining in zebrafish to a commercial goat anti-secretagogin antibody". Please give the manufacturer and catalog number of the tested antibody.

We added the relevant information in table 1 as well as RRID in the antibody paragraph in the methods and to the title page.

PLEASE NOTE: If the research in this article has been funded in whole or in part by the NIH, HHMI, and/or the Wellcome Trust, please be sure that the box for this is correctly checked in the Manuscript Details section on Manuscript Central, to assure that your article is deposited to PubMed correctly. Please check with ALL authors to ensure no relevant grants are overlooked.

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Thank you for your submission to Journal of Comparative Neurology.

Sincerely,

Dr. Thomas Finger
Associate Editor, Journal of Comparative Neurology

Dr. Patrick Hof
Editor-in-Chief, Journal of Comparative Neurology

You can upload your revised manuscript and submit it through your Author Center. Log into <https://protect-au.mimecast.com/s/YZ52BmimK8O1sO?domain=mc.manuscriptcentral.com> and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions."

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IMPORTANT: We have your original files. When submitting (uploading) your revised manuscript, please delete the file(s) that you wish to replace and then upload the revised file(s).

Reviewer: 1

Comments for the Authors

The manuscript demonstrates the requirement for the Nr4a2a orphan nuclear receptor in the terminal differentiation of dopaminergic retinal amacrine cells in zebrafish. Dopaminergic amacrine cells are also GABAergic. Nr4a2a also plays a role in the development of other select populations of GABAergic retinal amacrine cells as well. Interestingly many well studied types, such as amacrine cells immunoreactive for calretinin, calbindin or parvalbumin are not among these. The rewritten manuscript is much improved and the figures vary from good to brilliant.

Minor corrections:

Abstract:

'understand the interaction of various genes and assess the context in which Nr4a2 functions to refine its use for regenerative approaches.'

The reviewer didn't understand the phrase.

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We rephrased this, it now states:

"It can be utilised to assess consequences of gene manipulations and understand whether Nr4a2 only carries out its role in the presence of certain co-expressed genes. This will allow Nr4a2 use to be refined for regenerative approaches."

We had to make some additional minor deletions to ensure the abstract length was 250 words.

Results:

The section 'Tg(nr4a2a:eGFP) zebrafish line generation' replicates information provided in Methods section 'Transgenic zebrafish generation and screening.'

We shortened the methods to reduce replication of information.

Figure 10C: The bar graphs in different shades of green are artistic, but in my print, no difference could be discerned between dark green (weak fluorescence) and black (no fluorescence). The reviewer recommends more contrasting fills in the bars.

We've adjusted shading to utilise the whole range from white to black.

Conclusions:

'Following the expression analysis presented here, we can now gain a better understanding of the role of Nr4a2 in relation to DA neuron generation, for which this orphan receptor is best known for' Too many for's.

This has been rephrased and now states:

"Following the expression analysis presented here, we can now gain a better understanding of the role of Nr4a2 using the advantages of the zebrafish model to visualise and follow these developing cells *in vivo*. It will be particularly useful to characterise its specific role in DA neurons, as the Nr4a2 orphan receptor has already been implicated in DA neurogenesis."

At one paragraph, the discussion is a bit short.

The concluding paragraph is indeed one paragraph, the entire discussion spans 5 separate sections over 6 pages.

In vivo expression of Nurr1 / Nr4a2a in developing retinal amacrine subtypes
in zebrafish *Tg(nr4a2a:eGFP)* transgenics

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Abstract

The Nuclear receptor subfamily 4 group A member 2 (Nr4a2) is crucial for the formation or maintenance of dopaminergic neurons in the central nervous system including the retina, where dopaminergic amacrine cells contribute to visual function. Little is known about which cells express Nr4a2 at which developmental stage. Furthermore, whether Nr4a2 functions in combination with other genes is ~~also~~ poorly understood. Thus, we generated a novel transgenic to visualise Nr4a2 expression *in vivo* during zebrafish retinogenesis. A 4.1 kb fragment of the *nr4a2a* promoter was used to drive green fluorescent protein expression in this Tg(*nr4a2a:eGFP*) line. *In situ* hybridisation ~~experiments~~ showed that transgene expression follows endogenous RNA expression at a cellular level. Temporal expression and lineages ~~s~~ ~~analysis was~~ ~~were~~ quantified using *in vivo* time-lapse imaging in ~~zebrafish~~ embryos. Nr4a2 expressing retinal subtypes were characterised immunohistochemically. Nr4a2a:eGFP labelled multiple neuron subtypes including 24.5% of all amacrine interneurons. Nr4a2a:eGFP labels all tyrosine hydroxylase labelled dopaminergic amacrine cells, and other non-dopaminergic GABAergic amacrine populations. Nr4a2a:eGFP is confined to a specific progenitor lineage identified by sequential expression of the bhlh transcription factor Atonal7 (Atoh7) and Pancreas transcription factor 1a (Ptf1a), and labels post-mitotic post-migratory amacrine cells. Thus, developmental Nr4a2a expression indicates a role during late differentiation ~~stages within~~ of specific amacrine interneurons. Tg(*nr4a2a:eGFP*) is an early marker of ~~specific distinct~~ neurons including dopaminergic amacrine cells. It can be utilised to assess consequences of gene manipulations ~~to and~~ understand ~~the whether Nr4a2 only carries out its role in the presence of specific co-expressed genes. interaction of various genes and assess the context in which Nr4a2 functions to~~ This will allow Nr4a2 use to be refined ~~its use~~ for regenerative approaches.

Background

Within the retina the five main types of neurons can be subdivided into many subtypes that form distinct circuits to analyse different aspects of vision in parallel. The most diverse population are amacrine interneurons, which synapse onto and modulate signals between bipolar and ganglion cells in the inner plexiform layer of the retina (Masland, 1988). Dopaminergic interplexiform or amacrine cells are present in all vertebrate retinas (reviewed in Witkovsky, 2004; reviewed in Popova, 2014). While these retinal subtypes form specific connections including GABAergic synapses (releasing the neurotransmitter gamma-aminobutyric acid) onto various distinct post-synaptic neurons (Contini and Raviola, 2003; Volgyi et al., 2014; Debertin et al., 2015), dopamine itself acts as a paracrine neuromodulator, which can affect all retinal neurons via different G-protein coupled dopamine receptors (Contini and Raviola, 2003; reviewed in Popova, 2014; Hirasawa et al., 2015). Dopamine synthesis and release is modulated by light levels through cone and rod photoreceptors and melanopsin ganglion cells, as well as circadian rhythms (Sakamoto et al., 2005; Cameron et al., 2009; Van Hook et al., 2012; Zhang et al., 2012; reviewed in Popova, 2014; Vuong et al., 2015; Qiao et al., 2016). Although different experimental setups and species have yielded different data on its precise action, dopamine certainly affects visual circuits involved in light adaptation, colour discrimination, contrast sensitivity, acuity and circadian rhythms, as well as playing important trophic roles including eye growth (reviewed in Witkovsky, 2004; Jackson et al., 2012; Feldkaemper and Schaeffel, 2013; Ventura et al., 2013; Kim et al., 2014; Nebbioso et al., 2014; reviewed in Popova, 2014).

The proper generation of DAC like their DA counterparts across other brain areas is crucial for normal neurological functioning. The orphan nuclear hormone receptor Nurr1 / Nr4a2 first identified in 1992 (Law et al., 1992) plays an important role in the development of DA neurons across the central nervous system. Nr4a2 has been of particular interest, as its loss affects terminal

differentiation and survival of DA neurons in the substantia nigra (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Le et al., 1999; reviewed in Smidt and Burbach, 2009) and as it has been linked to Parkinson's disorder (Le et al., 2003; Liu et al., 2012a; Liu et al., 2012b).

Key differentiation factors including dopamine transporter and tyrosine hydroxylase (TH) are regulated through Nr4a2 acting synergistically with factors including Pitx3 and Foxa2 (reviewed in Arenas, 2005; Martinat et al., 2006; Jacobs et al., 2009; reviewed in Smidt and Burbach, 2009; Lee et al., 2010). These studies suggest that co-operation of different transcription factors may explain, at least in part, how subtype specification is achieved, even though individual factors themselves are expressed more broadly (Martinat et al., 2006). Identified pathways that regulate Nr4a2 include the Wnt signalling pathway through β -catenin binding of Nr4a2 (reviewed in Smidt and Burbach, 2009) and retinoid signalling (Volakakis et al., 2009).

In the zebrafish, two paralogues (a and b) with overlapping expression patterns have been identified, with Nr4a2a being more homologous to the Nr4a2 in higher vertebrates (Blin et al., 2008). *Nr4a2a* knockdown in zebrafish also showed the more drastic loss of DA phenotype in different CNS regions, equivalent to the phenotype obtained from knocking down both paralogues simultaneously, whilst *nr4a2b* knockdown did not display as severe a phenotype (Filippi et al., 2007). Thus, these paralogues act non-redundantly, with the *nr4a2a* paralogue most closely mirroring the described function of Nr4a2 in mammalian vertebrates.

In the vertebrate retina, Nr4a2 is expressed in a subpopulation of amacrine cells, including the DA subtypes (Filippi et al., 2007) and other GABAergic amacrine subtypes (Jiang and Xiang, 2009). Dominant-negative forms of Nr4a2 in the mouse and morpholino knockdown of Nr4a2a, but not Nr4a2b in the zebrafish results in an absence of DAC (Filippi et al., 2007; Jiang and Xiang, 2009). Misexpression in mouse has been shown to promote GABAergic amacrine differentiation. These studies suggest that in the retina, Nr4a2 may have a general role in the development of GABAergic including DAC.

In order to investigate further the role and expression profile of Nr4a2a during development of these retinal cell types, we generated a transgenic line that faithfully recapitulates the expression of endogenous *nr4a2a* RNA. By using enhanced green fluorescent reporter protein (eGFP), we are able to follow cells as they continue to mature and differentiate, assessing their final fate in terms of distribution and subtype composition, even though these cells may express *nr4a2a* only transiently during development. We confirmed that many retinal neurons belonging to multiple subtypes, which include all DA amacrine cells, express Nr4a2a. The genetic context (lineage) and temporal expression of Nr4a2a was characterised to elucidate during which stage of neurogenesis Nr4a2a function is most important. Our data shows that Nr4a2 expression in amacrine cells occurs only for a brief period during early post-mitotic differentiation, and that distinct AC subtypes express Nr4a2a during their development.

Methods

Animals

Zebrafish were maintained and bred at 26.5°C, and embryos of either gender used for experiments were raised at 28.5°C and staged as previously described (Kimmel et al., 1995) in hours postfertilisation (hpf). All procedures were carried out according to the provisions of the Australian National Health and Medical Research Council code of practice for the care and use of animals and were approved by the institutional ethics committee at the University of Cambridge, Monash University and University of Melbourne.

Transgenic construct cloning

A 4094 bp fragment of the zebrafish *nr4a2a* promoter region was PCR amplified from the BAC clone RP71-49K16 (Children's Hospital Oakland Research Institute, Oakland, USA) using advantage taq (Contech, 639201). Forward primer: 5'-GGGGACAACCTTTGTATAGAAAAGTTGCCCCCTCTATCCCTGTCACACA-3' and reverse primer: 5'-GGGACTGCTTTTTTGTACAAAGCTGGCTGCAATTAAACAAA-3'. The full *nr4a2a*:eGFPpA construct was generated using the tol2kit. The PCR product was recombined into the pDONRP4-P1R gateway entry clone, sequence verified and recombined via LR reaction with gateway entry clones pDEST vector containing tol2. The pME-eGFP and p3E-polyA entry clones were kindly provided by Dr K Kwan and Professor CB. Chien (Kwan et al., 2007).

Transgenic zebrafish generation and screening

The transgene construct (50 ng / μ l) was injected into the cell of one-cell stage WT zebrafish embryos together with (50 ng / μ l) Tol2 transposase mRNA (1 – 2 nl / embryo). Transposase mRNA was synthesised using mMessage mMACHINE (Amersham). [Screening of transgenic](#)

zebrafish throughout multiple outcrossed generations is described in the results section. About 800 embryos with mosaic eGFP expression at 2–5 days postfertilisation (dpf) were grown and screened for potential founders with germline transmission 100–500 offspring from 27 putative founders were screened at 2, 3, 4 and 5 days postfertilisation. Nine founders were identified with offspring that showed eGFP reporter expression in the expected regions and F1 were raised. Each F1 fish was outcrossed with WT and expression screened at 5 dpf using immunohistochemistry with positively stained F2 families subsequently raised. Offspring of subsequent generations were used for detailed expression screening at 2, 3, 4, 5 dpf.

Immunohistochemistry

Zebrafish embryos and larvae were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline pH 7.4 (PBS) at room temperature (RT) for 3 hrs or overnight at 4°C. Zebrafish were rinsed, cryoprotected in 30% sucrose, embedded in OCT (Tissuetek) and cryosectioned at 14 µm thickness. All immunohistochemistry steps were performed at RT.

Sections were incubated in blocking buffer (5% foetal calf serum, 1% bovine serum albumin, 0.5% Triton X-100 in PBS) for 30 min. Sections were incubated in primary antibodies diluted in blocking buffer overnight. Primary antibodies used in this study are listed in Table 1. Sections were rinsed in PBS and incubated in secondary antibodies for 1 hour at RT. Secondary antibodies diluted in blocking buffer were goat or donkey anti-mouse, anti-rabbit or anti-goat IgG conjugated to Alexa 488 or 546 fluorophores (1:500 dilution, Molecular Probes). After further rinses, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and sections were coverslipped in Mowiol.

Antibody characterisation

The mouse anti-tyrosine hydroxylase antibody (RRID: AB_2201528) is used here to specifically label dopaminergic neurons. The antibody is raised against a rate-limiting enzyme during dopamine production. This commercial antibody was raised against TH purified from rat PC12 cells. It recognises an epitope outside of the regulatory N-terminus, and a 62 kDa band in immunoblots of zebrafish brain (Uytenbroek et al., 2010). In zebrafish CNS this antibody exclusively labels TH1 as shown by co-localisation with *th1*, but not *th2* *in situ* hybridisation (Chen et al., 2009). Additionally, immunolabelling with this antibody is abolished following morpholino mediated knockdown specifically of Th1 in embryonic zebrafish CNS (Kuscha et al., 2012).

The rabbit anti-gamma-aminobutyric acid antibody (RRID: AB_477652) was used to label a large subpopulation of amacrine cells, which express the GABA neurotransmitter. The antibody specifically recognises GABA in a dot blot assay (manufacturer's datasheet, species not mentioned). The structure of GABA is identical in all vertebrate species. Within the zebrafish retina, the staining pattern obtained using this GABA antibody is comparable with that obtained using antibodies against GAD65 and GAD67, the glutamic acid decarboxylates, which are the enzymes that convert glutamate to GABA (Connaughton et al., 1999).

The rabbit anti-calbindin D-28K antibody (RRID:AB_213554) specifically recognises the expected 28 kDa band in Western blots in zebrafish brain homogenate. Calbindin immunoreactivity in zebrafish is abolished when the primary antibody is preabsorbed with recombinant rat calbindin (Uytenbroek et al., 2010).

The rabbit anti-calretinin antibody (RRID: AB_2068506) specifically recognises a 28kDa Western blot band in zebrafish brain homogenate (Uytenbroek et al., 2010).

The goat anti-choline acetyl transferase antibody (RRID: AB_2079751) recognises a specific 70 kDa band on Western blots of mouse brain lysate (manufacturer's datasheet). In zebrafish, immunoreactivity is abolished when the antibody is preabsorbed with recombinant rat ChAT (Olsson et al., 2008).

The chicken anti-green fluorescent protein antibody (RRID: AB_2534023) was directly raised against GFP isolated from jellyfish (*Aequorea victoria*) and the IgY fraction was purified by affinity purification (manufacturer's datasheet). The antibody recognised purified GFP consisting of 238 amino acids at 27 kDa (Hamamoto et al., 2016). The GFP transgene is unrelated to any zebrafish proteins and staining of GFP in non-transgenic zebrafish results in no immunoreactivity (data not shown).

The rabbit anti-neuropeptide Y antibody (RRID: AB_572253) specifically stains NY in the rat central nervous system, which is blocked by preabsorption (manufacturer's datasheet). NY is a small 36 amino acid neurotransmitter that is highly conserved across all vertebrates, with zebrafish NY showing 89% homology to the human NY (Soderberg et al., 2000). In goldfish, preabsorption of this antibody with rat NY abolishes immunoreactivity in the brain and pituitary as shown by immunoblotting, enzyme immunoassay and radioassay (Matsuda et al., 2009).

The mouse anti-parvalbumin antibody (RRID: AB_2174013) specifically stains Ca²⁺ bound forms of parvalbumin and recognises a 12 kDa protein in Western blots of mouse brain lysate (manufacturer's datasheet) and whole zebrafish homogenate (our data not shown).

The rabbit anti-secretagogin antibody (RRID: AB_2034060) used shows identical staining in zebrafish to a commercial ~~goat~~-sheep anti-secretagogin antibody not used for analysis in this study (data not shown, [RRID: AB_2034062](#)). In mammalian species both of these antibodies label the same cells as a non-commercial secretagogin antibody that recognise the 32 kDa predicted band in mouse retina and cerebellum (Puthussery et al., 2010; Weltzien et al., 2014).

The rabbit anti-serotonin antibody (RRID: AB_477522) reacts with serotonin-containing fibers in rat brain and specific staining is inhibited by preabsorption with serotonin or serotonin-BSA (manufacturer's datasheet). 5-HT is identical in all vertebrate species (Olsson et al., 2008). Preabsorption with 5HT- BSA conjugate completely abolishes zebrafish staining (Uytenbroek et al., 2010).

Wholemout and section in situ hybridisation

In situ RNA hybridisation was performed following standard protocols for wholemount embryos (Thisse and Thisse, 2008) or cryostat sections (Butler et al., 2001). Constructs for *nr4a2a* RNA probes were kindly provided by Professor Driever. For antisense RNA probe, DNA was linearised with XhoI and transcribed with SP6 RNA polymerase.

Embryos were fixed in 4% PFA overnight, rinsed and processed as wholemounts or cryoprotected in 30% sucrose, embedded in OCT and cryostat sectioned at 20 µm thickness.

Wholemout embryos were dehydrated serially into 100% methanol, rehydrated into PBST (0.1% Tween 20 in PBS), digested in proteinase K (0.25 – 25 µg/mL depending on age) for 25 minutes, postfixed in 4% PFA, prehybridised at 65°C for 2 – 6 hours and hybridised with RNA probe overnight at 65°C. Unbound probe was removed by 65°C serial dilution from 50% formamide / 2X SSC through 2X SSC, then 0.2X SSC and finally into PBST at room temperature. Embryos were blocked (2% sheep serum, 5% bovine serum albumin in PBST) incubated in preabsorbed anti-Dig Fab fragment antibody (1:100) overnight, rinsed, equilibrated in staining buffer (100 mM NaCl, 0.1% Tween 20 in 0.1M Tris pH 9.5) and stained with NBT/BCIP (Roche Diagnostics 11681451001). After postfixation with 4% PFA, embryos were equilibrated into 100% glycerol and imaged.

For section *in situ* hybridisation, sections were postfixed with 4% PFA, rinsed in 2x SSPE, incubated 0.1M triethanolamine without and then with 0.25% acetic anhydride, rinsed, prehybridised at 60°C for 2 – 5 hours followed by probe hybridisation overnight at 60°C.

Sections were rinsed in 2x SSPE at RT, 20 µg/ml RNase A in 4x SSPE at 37°C for 30 minutes and 50% formamide in 2x SSPE at 60°C for 45 minutes. After rinses in 2x SSPE at and buffer 1 (150 mM NaCl in 0.1M Tris pH 7.5) at room temperature, sections were blocked in 0.5% blocking reagent (Roche) in buffer1 for 2 hours and incubated in preabsorbed anti-Dig Fab fragment (1:100) for 1.5 hours. Antibody was rinsed off with buffer 1, equilibrated in staining buffer (100 mM NaCl

in 0.1M Tris pH 9.5) and signal was visualised with NBT/BCIP or Fast Red (Sigma, F4648) followed by coverslipping in Mowiol.

Imaging of fixed and live embryos

Wholemout fixed transgenic embryos were imaged on the SZX2-ILLB stereo microscope (Olympus) using cellSense Standard software (Olympus). Wholemount *in situ* hybridisation stained embryos were imaged on a BX51 microscope with dotSlide software (Olympus). Sections were photographed at the Z1 Axioscope using Axiovision software (Zeiss) using the Apotome. Images were processed in Adobe Photoshop (brightness and contrast adjustments) and figure panels combined in Adobe Illustrator.

For *live* time-lapse imaging, embryos were treated with 0.002% phenylthiourea (PTU) from 24 hpf onwards to delay eye pigment formation, and embedded in 1% low melt agarose / 0.002% PTU / 0.04% tricaine methane sulfonate in E3 embryo medium (Jusuf et al., 2013). Imaging was performed with a confocal system (Zeiss LSM5/710 meta) using a water immersion 20X objective and 488 nm argon (GFP) and 560 nm laser lines (RFP). Optical sections (120 – 150 μm) of 3 μm thickness were taken through the eye every 30 min for 24 hours. Image data were acquired using Zeiss ZEN software and processed using Imaris and Fiji. Brightness and contrast were adjusted in Adobe Photoshop.

Analysis

Numbers of embryos / cells analysed are indicated in the relevant results section.

Quantification of the percentage of retinal amacrine cells expressing Nr4a2a:eGFP was performed using rectangular regions of interest (ROIs) that were 50 μm wide and encompassed ~ 40 amacrine cells per ROI.

The brightness intensity of transgene expression in individual cells was quantified in Fiji ($n = 251$ cells, $n = 6$ embryos). A small rectangular ROI within the centre of each cell (with dimensions about half of the cell body) was chosen and intensity measured using the “Colour histogram” analysis tool (0 – 255). This measure is independent of the size of the ROI, provided the ROI remains within the boundaries of the cell.

Co-labelling of Nr4a2a:eGFP expression in retinal amacrine subtypes / subpopulations were quantified in sections of 5 dpf retinas. The number of cells analysed for each are indicated in the relevant section. For markers that label a relatively dense population of cells, images were randomly chosen by arbitrarily choosing a section with or close to the optic nerve section, though the co-labelling pattern should be the same across the entire retina. For markers that label a relatively small population of cells, the number of embryos sampled was increased to ensure sufficient cell number for analysis and every section containing a labelled cell was imaged and included in analysis. Analysis was conducted relative to the marker, i.e. how many immunolabelled cells expressed Nr4a2a:eGFP and at which intensity.

Results

Tg(nr4a2a:eGFP) zebrafish line generation

~~The cloned transgene was injected into the cell of one-cell stage~~Injected WT eggs and zebrafish embryos ~~were~~ screened for mosaic GFP expression at 2 – 5 days postfertilisation (dpf) ~~resulted in -a~~ Approximately 800 ~~identified~~ embryos ~~were~~ grown to adulthood. Offspring (n = ~100 – 500) of 27 putative founders were screened for pattern of eGFP expression at 2, 3, 4 and 5 days postfertilisation (dpf). We identified germ-line transmission in 17 of 27 screened founder fish. Nine of these founders showed eGFP reporter expression in the expected central nervous system (CNS) regions and F1 families were raised of these. Each of the F1 fish was outcrossed with WT and F2 offspring were screened for Nr4a2a:eGFP expression. We identified five independent outcrossed F1 fish from three original injected F0 fish (ID14, 26 and 27) that showed the expected pattern of Nr4a2a:eGFP expression in the retina. These five founders showed comparable and stable expression patterns. Outcrossed offspring raised to the F3 or F4 generation were used for further characterisation including time-series of Nr4a2a:eGFP expression in wholemount *live* larvae and comparison to *in situ* RNA signal.

Temporal transgene expression follows endogenous RNA in situ pattern

We characterised the expression pattern of the eGFP reporter transgene at key developmental ages, at which strong endogenous *nr4a2a* RNA *in situ* hybridisation signal was previously described (Filippi et al., 2007). At 48 hpf, strong *nr4a2a* promoter driven eGFP expression was observed in the head and spinal cord (Figure 1A – D).

At this age, the *nr4a2a* RNA signal is expressed in the different CNS regions including the retina and spinal cord (Figure 1A-D) as well as ventral telencephalon, diencephalon, tegmentum and medulla oblongata consistent with previous descriptions (Filippi et al., 2007; Blin et al., 2008). By 72 hpf and 96 hpf the endogenous expression of *nr4a2a* RNA is more restricted in many regions

including spinal cord and hindbrain (black arrowheads, Figure 1), in which Nr4a2a might be particularly important only during developmental stages of these structures. The transgenic line shows strong eGFP expression throughout the CNS and is maintained in regions that have now started downregulating endogenous expression (white arrowheads, Figure 1), due to the perdurance of the eGFP transgene with half-life of > 1 day (Andersen et al., 1998; Corish and Tyler-Smith, 1999) (Figure 1E – L).

A detailed time series was conducted to follow the onset of the eGFP signal during these early stages of CNS development. Strong reporter protein expression could be first visualised at 24 hpf and 30 hpf in the telencephalon (Figure 2A, B). Transgene eGFP was rapidly upregulated across the CNS between 36 hpf – 48 hpf, such as the midbrain and the hindbrain, and continued in spinal cord cells (Figure 2C – E). Between 48 – 60 hpf, eGFP expression was relatively stable and remained strong in all areas (Figure 2F, G). At this age, retinal expression of Nr4a2a:eGFP can be seen (higher power inset Figure 2G). Thus, cells that turn on Nr4a2a:eGFP in the expected CNS areas maintain stable eGFP expression.

Nr4a2a:eGFP labelled cells co-label with endogenous nr4a2a RNA in the retina

Similarly, the expression of *nr4a2a* RNA *in situ* signal and Nr4a2a:eGFP transgene expression was compared in retinal sections of 5 dpf embryos, where individual cells could be identified (Figure 3A – B''). By this stage the central retina has been fully developed and functional for at least 24 hours. In the central retina, co-localisation with the eGFP transgene signal could be seen for 82% of cells also labelled with *nr4a2a* RNA *in situ* signal (n = 155 cells, asterisks in inserts, Figure 3a – a'', B – B''). The immunohistochemical recovery of the GFP signal is variable after *in situ* hybridisation resulting in fewer and generally weaker GFP signal after such procedures (Figure 3). By 5 dpf, there were additional neurons positive only for Nr4a2a:eGFP in these regions. This is consistent with expression of our transgenes in cells that still express *nr4a2a* mRNA additional to those that have meanwhile switched off *nr4a2a* mRNA expression. The eGFP was

specifically chosen to provide a strong label allowing us to follow cells in which endogenous mRNA as well as protein may or may no longer be expressed in at the time of analysis.

Nr4a2a:eGFP expression recapitulates temporal and spatial pattern of the endogenous RNA expression

A comparative time-series of endogenous *nr4a2a* RNA expression by *in situ* hybridisation and *Tg(nr4a2a:eGFP)* transgene expression was conducted in the retina. At 28 hpf when the first progenitors exit the cell cycle to start generating the first born retinal neurons (retinal ganglion cells), there is neither expression of endogenous *nr4a2a* RNA nor GFP transgene (Figure 4A, H). Amacrine interneurons first start differentiating at 35 hpf as they turn on expression of Pancreas transcription factor 1 a (Ptf1a) after multipotent progenitors undergo their final apical division and start migrating down to the future amacrine cell layer (ACL) in the inner nuclear layer (INL), which forms in the middle of the retina (Jusuf et al., 2009). At 40 hpf, we first observe cytoplasmic *nr4a2a* RNA expression in the centre of the retina, where the future amacrine layer is forming (Figure 4B). No labelled nuclei are ever observed more apically, suggesting that *nr4a2a* expression is activated only once cells that have migrated to their final location. At this time-point the *Nr4a2a:eGFP* transgene is only detected very rarely in a few cells closest to the lens (Figure 4I). By 48 hpf, when endogenous RNA expression and transgene expression is strong in the ACL (Figure 4C), eGFP positive cells are additionally observed in the ganglion cell layer (Figure 4J). At 60 hpf the endogenous RNA *in situ* signal starts to be downregulated in the ACL closest to the inner plexiform layer (Figure 4D). Finally, endogenous *nr4a2a* RNA expression becomes further restricted to only a few amacrine cells by 120 hpf (Figure 4E – G). The strongest *Nr4a2a:eGFP* expression at these time-points is similarly in the ACL (Figure 4L – N). The subsequent loss of endogenous mRNA staining suggests that *Nr4a2a* may be particularly important during a specific period of development, whereas the maintenance of *in situ* signal for *nr4a2a* mRNA in a few specific cells might point towards a secondary role in maintenance or survival of a subset of these neurons. The density and distribution of the cells expressing the endogenous *nr4a2a* mRNA at 120 hpf (Figure

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4G) is similar to the pattern observed with antibody staining for dopaminergic neurons at this stage (Figure 6).

In the inner nuclear layer Nr4a2a:eGFP is expressed exclusively in post-mitotic and post-migratory amacrine interneurons

Using the Tg(*nr4a2a:eGFP*) line, we performed *in vivo* time-lapse imaging experiments to correlate the timing of transgene expression to the different stages of neural differentiation. Ptf1a is a transcription factor that is expressed transiently during the development of all amacrine cells (Jusuf et al., 2011), of which DA neurons are a subtype. Using a double transgenic line additionally expressing RFP under the *ptf1a* promoter, we can visualise inhibitory neurons just after they undergo their last progenitor division apically and migrate to the forming amacrine layer (Figure 5A - H). As previously described, Ptf1a:RFP can be first detected around 35 hpf, when amacrine cells start to differentiate (Jusuf and Harris, 2009). At this time, the Nr4a2a:eGFP transgene is just starting to be detected faintly in the ganglion cell layer in Ptf1a unlabelled ganglion cells (Figure 5A). By 41 hpf, Nr4a2a:eGFP transgene can be visualised in the ACL (Figure 5D), suggesting it is first expressed about 5 – 6 hours after Ptf1a expression, and consistent with our fixed samples, only in cells that have already finished migrating and forming the future amacrine layer. The vast majority of cells that have just started expressing Nr4a2a:eGFP in the INL are expressing Ptf1a:RFP (93%, n = 358 cells, 14 images from 5 time-lapse movies), consistent with specification as amacrine interneurons (Figure 5I, white dots mark cells co-expressing Nr4a2a:eGFP and Ptf1a:RFP). Ptf1a:RFP labelled horizontal cells that start migrating back apically around 45 hpf never express Nr4a2a:eGFP (Figure 5F - H). These time-lapse experiments identify the expression of Nr4a2 in post-mitotic post-migratory neurons, indicating that its role is confined to later stages of neurogenesis that might include subtype fate specification and other terminal differentiation processes. Additionally, Nr4a2a is expressed in a specific subpopulation of Ptf1a expressing neurons that includes some amacrine, but not horizontal cells.

Post-mitotic Nr4a2a:eGFP expressing retinal neurons in all layers arise exclusively from the Atonal 7 lineage

As seen in our fixed tissue (Figure 4I – N) and the time-lapse imaging (Figure 5A – H), we unexpectedly observed Nr4a2a:eGFP labelling in the ganglion cell layer, which has not been described for zebrafish retina previously. The intensity of the transgene, which might be a qualitative indicator of promoter activation is variable in cells within this layer, though usually weaker than the strongest expressing cells in the ACL, once they upregulate their expression (Figure 4L – M). Although most of the endogenous RNA *in situ* signal was exclusively observed in the forming ACL in the INL, we did come across a few examples of RNA expressing cells in the GCL in both in wholemount *in situ* preparations (arrows in Figure 6C – E) as well as in sections processed for *in situ* hybridisation (arrowhead in high magnification insets (boxed) in Figure 6F – G). This would be consistent with very transient (and possibly weak) expression of *nr4a2a* mRNA within some of these cells, which remain labelled by our stable strong eGFP expression within this layer.

We used the Tg(*atoh7:RFP*) line to characterise the extent to which Nr4a2a:eGFP positive cells were related to the progenitor lineage marked by the transcription factor Atoh7, which is upregulated to specify ganglion cell fates and downregulated after very transient expression in cells destined to give rise to photoreceptors, horizontal cells and a subpopulation of amacrine cells. Atoh7:RFP is first detected at 28 hpf, just prior to the terminal mitosis of the first born ganglion cells (Poggi et al., 2005). The first Nr4a2a:eGFP positive cells are seen 7 hours later and co-label with the Atoh7:RFP transgene in the ganglion cell layer (Figure 7). Subsequent expression of Nr4a2a:eGFP in the inner nuclear layer where amacrine cells are found also co-labels with Atoh7:RFP (total 98%, n = 646 cells, 19 images from 7 time-lapse movies). Thus Nr4a2 expression in any of the retinal layers only occurs in cells from the Atoh7 lineage. Taken together, these time-lapse experiments identify Nr4a2 expression in a specific subpopulation of retinal neurons, namely those that arise from a neural lineage characterised by sequential expression of Atoh7 and Ptfla..

Dopaminergic neurons within the retina express Nr4a2a:eGFP

Since Nr4a2 has been implicated in generating DA neurons, we first compared the expression of tyrosine hydroxylase protein against our eGFP transgene using immunohistochemistry to ascertain whether the lineage specific subpopulation of retinal neurons include the DAC. Co-staining of tyrosine hydroxylase with *nr4a2a in situ* RNA signal in previous studies already revealed some, but not complete co-labelling (Filippi et al., 2007; Blin et al., 2008). With the perdurant GFP in our transgenic line, we can now assess whether these cells truly never expressed Nr4a2 or only transiently expressed *nr4a2a* RNA.

Tyrosine hydroxylase immunoreactive cells co-labelled with our eGFP transgene in the retina (100%, n = 88 cells from 39 embryos), though unexpectedly not necessarily at the highest transgene intensity level (only 3% expressed the transgene at a high level compared to 53% weakly and 43% at medium brightness, Figure 8A – C, Figure 10C), suggesting that these are not the cells that generate the most Nr4a2a. Nonetheless, these results show that DAC interneurons in the retina arise from cells that express Nr4a2a during their development.

Nr4a2a:eGFP expression in distinct non-dopaminergic retinal amacrine cells

Nr4a2 is expressed in more retinal neurons that are not labelled by dopaminergic markers such as tyrosine hydroxylase. Within the amacrine cell half of the INL (distinguishable by weaker DAPI labelling compared to the more intensely labelled bipolar cell half), Nr4a2a:eGFP is expressed in 24.5% of amacrine cells (n = 509 from 13 zebrafish). Using our transgenic line in combination with established amacrine subtypes or subpopulation (possibly multiple subtypes) markers we further characterised, if Nr4a2a was expressed in specific amacrine subpopulations.

Conserved with reports in other vertebrates, in the zebrafish retina, Nr4a2 is expressed in the majority of GABAergic amacrine subtype (64%, n = 1351 cells from 12 zebrafish), which include

DACs. The intensity of the transgene varied widely, with 18% of GABAergic cells expressing Nr4a2a:eGFP strongly, 24% at medium levels and 22% at weak levels. However, not all GABAergic amacrine cells show Nr4a2 expression (36% did not co-localise), suggesting it is not necessary for the global GABAergic neurotransmitter phenotype (Figures 9A).

In contrast, all Nr4a2a:eGFP expressing retinal cells also expressed GABA. Using other amacrine subtypes markers Nr4a2 was distinctly expressed in amacrine population that primarily did not co-label with any of these markers (asterisks Figure 9B – H). This included antibodies against the calcium binding proteins calretinin (16% co-localisation, n = 1322 cells from 15 zebrafish), calbindin (10% co-localisation, n = 598 cells from 17 zebrafish) and parvalbumin (25% co-localisation in the INL, n = 545 cells from 17 zebrafish, 4% co-localisation in the GCL, n = 724 cells from 11 zebrafish), as well as subtype specific markers against secretagogin (22% co-localisation, n = 589 cells from 17 zebrafish), choline acetyltransferase (22%, n = 232 cells from 6 zebrafish), neuropeptide Y (9% co-localisation, n = 77 cells from 30 zebrafish) or serotonin (4% co-localisation, n = 200 cells from 14 zebrafish).

Because the transgenic line showed differences in transgene brightness intensities, correlation of subjective categorization with quantitative brightness measures was performed (Figure 10A, B). Cells were assigned into one of four categories: No co-label or co-labelling with weak, medium or strong GFP expression (Figure 10). The brightness of each cell was then quantified (Fiji) and plotted. Three relatively distinct populations with separate peaks could be identified, which corresponded well to subjective categorisation. Brighter labelling can be due to the Nr4a2 promoter either being activated more strongly or for a longer period, both of which could lead to an increase in the number of GFP (and presumably Nr4a2) proteins being generated. Subsequent co-labelling with amacrine markers such as those from Figures 8 and 9 were analysed according to such categories (Figure 10C, n = number of cells analysed for each immunohistochemical marker). This quantification confirmed that TH⁺ DACs all express Nr4a2a (though to varying degree, with the majority expressing Nr4a2 at medium levels). Secondly,

GABAergic amacrine cells (which account for about half of the zebrafish amacrine subtypes) showed substantial co-labelling with Nr4a2a:eGFP, though about a third did not show any detectable transgene labelling. Whether the different Nr4a2a:eGFP intensities correspond to distinct GABAergic subtypes within the GABAergic group could not be determined with available markers. Surprisingly none of the other markers showed much Nr4a2a:eGFP expression. This confirms that Nr4a2 is not randomly expressed to the same proportion within every amacrine subtype but selectively expressed in distinct amacrine subtypes, namely those that are not labelled with our available markers.

Discussion

The correct development of different neuron subtypes is crucial for the formation of neural circuits, each carrying out a distinct function. This is particularly well characterised in the vertebrate retina, where distinct subtypes of the five main types of neurons form specific synaptic circuits that analyse discrete sub-modalities of vision such as colour. Across the CNS most genetic and neurodegenerative disorders affect only specific subtypes. Thus, an understanding of the lineages and gene networks that generate different types of neurons during development forms the basis of understanding genetic neural disorders and direct approaches to recapitulate such developmental processes towards regenerating lost subtypes in the adult CNS. Here, we generated a novel transgenic tool to dissect out the genetic pathways that give rise to particularly (but not exclusively) the dopaminergic neuron subtypes, which include a dopaminergic amacrine interneuron subtype in all vertebrate retinas. Dopaminergic neurons are crucial for a number of catecholamine pathways, which when disrupted are linked to disorders such as Parkinson's or schizophrenia (Jankovic et al., 2005). Within the retina, dopamine released from interplexiform and amacrine cells acts as a paracrine neuromodulator and contributes to light adaptation and circadian rhythms, colour and contrast discrimination and visual acuity due to the role of dopaminergic retinal amacrine cells in this visual sub-modality (Witkovsky, 2004; Jackson et al., 2012; reviewed in Masland, 2012; reviewed in Popova, 2014).

Establishment of novel Tg(nr4a2a:eGFP) zebrafish line

A number of transgenic zebrafish lines exist that mark different populations of DA neurons driven by promoters of genes encoding for factors such as dopamine transporters, tyrosine hydroxylase, orthopedia homolog b (Gao et al., 2005; Meng et al., 2008; Wen et al., 2008; Bai and Burton, 2009; Fujimoto et al., 2011; Xi et al., 2011). These lines are extremely useful for labelling DA neurons, but if we are to understand how DA neurons are first generated or differentiated, it is

important to characterise the expression of genes that are involved in the earliest stages of DA neuron specification and differentiation. Here, we generated a novel transgenic line using the promoter of the *Nr4a2a* gene that is expressed very early in neuron development to be used in conjunction to the specific DA lines to visualise DA neurons from the very beginning. This allows us to perform time-lapse experiments, where cells that are identified to be dopaminergic by late differentiation markers at later time-points can then be tracked back in time and followed by expression of the eGFP in this transgenic line. Cells can be tracked in genetic studies, which affect early development and/or differentiation of neurons at stages at which they do not yet express the final DA machinery, including for example the dopamine transporter and tyrosine hydroxylase. Here, we utilised this line to study the expression of *Nr4a2a*. Alterations in *Nr4a2a* expression can be compared using gene knockout or overexpression studies within this population to clarify the causes of DA neuron loss. By following the transgenically labelled cells, we can assess cell fate, cell number, contributions of apoptosis, differentiation arrest and the features of DA neurons that rely on *Nr4a2a*. Understanding the dynamic expression of individual genes such as *Nr4a2a* may drive forward therapeutic treatment.

Within the retina, the fluorescent eGFP reporter expression matches the timeline of endogenous RNA expression and co-labels in individual retinal cells. As expected, our detailed time course analyses additionally shows eGFP labelled cells that do not show mRNA signal, revealing that the stable eGFP reporter can be visualised in cells that might only activate the endogenous *nr4a2a* promoter transiently. Consistently, we observe a gradual increase of cells labelled by our transgene in appropriate layers suggesting that the more stable eGFP perdures throughout retinogenesis. At 5 dpf, when visual circuits are functional (Easter and Nicola, 1996; 1997; Schmitt and Dowling, 1999), we still see little decrease in the number of eGFP cells, although we cannot rule out that a few cells may no longer express the transgene. Thus, all cells that express *Nr4a2a* throughout development can be followed. This includes the expression in the ganglion cell layer, which has also been observed in the *Tg(dat:EGFP)* zebrafish line (Xi et al., 2011) and the mouse

retina (Jiang and Xiang, 2009). While we find similar co-labelling of endogenous *nr4a2a* mRNA and the eGFP transgene as well as eGFP-only labelled cells in other CNS regions, the remainder of the discussion focuses only on the retina. A thorough time-course analysis of co-labelling for relevant CNS areas of interest should be conducted as required, to determine whether the transgene faithfully recapitulates *Nr4a2* expression in these regions.

Developmental lineage specific expression of Tg(nr4a2a:eGFP) in the retina

The expression of *nr4a2a* RNA and *Nr4a2a:eGFP* in the INL is detected after amacrine cell differentiation has begun (Jusuf and Harris, 2009). The time-lapse imaging confirms, that these cells have exited the cell cycle and completed migration to the amacrine layer. This is consistent with the lack of cell cycle labelling including 5-Bromo-2'-deoxyuridine DNA synthesis labelling, phospho-histone H3 M-phase marker, or proliferating cell nuclear antigen labelling of *Nr4a2* expressing cells in the rodent retina (Jiang and Xiang, 2009; Li et al., 2009; Li et al., 2012).

In contrast to previous zebrafish retinal *nr4a2a* expression data, a transgenic zebrafish line labelling differentiated DA neurons by driving reporter GFP under the dopamine transporter promoter also expressed in the GCL, though these might be displaced amacrine cells (see Figure 4B, 7A Xi et al., 2011). Another zebrafish line, in which a rat *th* promoter drives expression of a reporter GFP also shows widespread expression across most of the amacrine and ganglion cell layer, though how this can be reconciled with the much more limited TH antibody staining remains unclear (Gao et al., 2005). Consistent with our expression data, in the mouse retina, *nr4a2 in situ* signal was also observed more numerous in the GCL (Jiang and Xiang, 2009). We believe that transgenically labelled cells within the GCL faithfully recapitulate *nr4a2a* promoter activation, with our *in situ* expression indicating that *Nr4a2a* is expressed, though extremely transiently, in this layer.

Using *in vivo* time-lapse imaging we characterised a stereotypical lineage of *Nr4a2a* retinal neurons. *Nr4a2a:eGFP* expression in all retinal layers (*i.e.* inner nuclear layer and ganglion cell

layer) was exclusively within strongly labelled Atoh7 cells. Strong Atoh7 expression defines a specific progenitor subpopulation that gives rise to all ganglion cells as well as subpopulations of other neurons including amacrine cells (Poggi et al., 2005; Jusuf et al., 2011; Jusuf et al., 2012). Nr4a2a:eGFP expression specifically in the inner nuclear layer additionally only occurred in cells from the Ptf1a lineage. Thus Nr4a2a expression is limited to a subpopulation of amacrine cells including those we previously showed to arise from the Atoh7 lineage (Jusuf et al., 2011).

● Ganglion and amacrine cells start differentiation in the zebrafish retina at 28 hpf and 35 hpf, respectively (Kay et al., 2001; Poggi et al., 2005; Jusuf and Harris, 2009), significantly before any *nr4a2a* mRNA or Nr4a2a:eGFP expression can be detected. This indicates that Nr4a2a is unlikely to play an important role in cell cycle exit and functions later at the stages of subtype diversification or terminal differentiation of neurons.

Nr4a2a:eGFP expressing in DA and non-DA neuron subtypes

Due to its implication for DA neurons, the main focus in the literature has been on the expression and role of Nr4a2 for this particular cell fate. Indeed we find that tyrosine hydroxylase immunoreactive cells (marker of DA neurons across all CNS regions) are indeed co-labelled with the Nr4a2a:eGFP in our line. Within the retina, we did not distinguish between the two described subpopulations of tyrosine hydroxylase immunoreactive cells (Jang et al., 2011), though our results are consistent with Nr4a2a expression in both of these populations. Additionally, Nr4a2 seems to be expressed in a distinct subpopulation of amacrine interneurons in the retina (rather than randomly in a similar percentage of each subtype). This was strikingly exemplified by the lack of Nr4a2 for most of the subtype markers tested. Beside TH, Nr4a2 cells in the retina seem to be exclusively expressed in other GABAergic cells, though not all GABAergic cells express Nr4a2. This pattern closely resembles the expression of the Barhl2 transcription factor, which is also confined to this lineage and can specifically drive GABAergic phenotype (Jusuf et al., 2012). It will be interesting to investigate the relationship in the expression of Nr4a2 and Barhl2 and assess whether they work

independently or together to specify these amacrine cell fates. In other CNS regions, we also observed Nr4a2 transgene expression primarily within GABAergic cells, though there were many more GABAergic cells that did not express Nr4a2. Thus, there may be region specific subtype distributions of Nr4a2.

We unexpectedly identified three relatively separate Nr4a2a expression profiles consisting of weak, medium or strong GFP expression rather than a normal distribution of a continuum of transgene expression, which could simply reflect expression variation. This was observed in every animal examined, and within a given animal, the three different transgene intensities identified subjectively correlated 100% with objective ImageJ measurements. The only small overlap observed in the graph in Figure 10 is due to differences between retinas, in which measurements of the brightest “weak” cell in one retina may be larger than the measurement of the dimmest “medium” cell in another retina. Differences between retinas could also arise if some animals are homozygous for GFP, i.e. leading to a relative brighter expression than in heterozygous animals. Given the limited subtype markers available to label amacrine populations in zebrafish, we were not able to identify a strong correlation between GFP intensity and specific subtype. It will be interesting to correlate intensity with different aspects of subtypes (not only immunohistochemical markers, but perhaps morphology) and to test whether the level of Nr4a2 expression (with brighter cells either activating transcription at a higher level or for a longer developmental period) has different functions during neurogenesis and perhaps distinct effects on different subtypes.

There are also many neurons across the central nervous system that express Nr4a2 in mammals and Nr4a2a/b in the zebrafish, but do not generate DA neurons (Filippi et al., 2007). This is at least partially explained by the co-ordinated role Nr4a2 is believed to play with other factors such as Pitx3 and Fox2a (reviewed in Smidt and Burbach, 2009; Lee et al., 2010). However, within the retina, the DAC do not express the same co-factors, such as Pitx3. Thus, the question remains, what the exact role of Nr4a2 is during development, subtype fate specification, maintenance or cell survival, noting that its role may differ depending on the CNS region. Given that Nr4a2a is

expressed early enough during development to influence all of these aspects of development and maintenance within the retinal neurons as we describe here, its expression in non-DA subpopulations of cells shows that Nr4a2a at least within this tissue is not sufficient to drive the full DA subtype fate. This may reflect additional factors being necessary or point towards the importance of maintaining its expression to prevent alternate fates. This is a particularly important result to consider given the regenerative efforts that are already using overexpression of Nr4a2 by itself to specifically drive DA neuron generation (Lei et al., 2011; Hong et al., 2014). Important co-factors, which may be CNS region-specific, need to be identified to drive these efforts further.

Conclusions

Nr4a2a is expressed in both DA and non-DA (primarily GABAergic) neurons in the vertebrate retina. By using a novel Nr4a2a:eGFP transgenic zebrafish line cells that express Nr4a2a at some stage of their development, even if only very transiently, were found to originate from a specific progenitor lineage. Following the expression analysis presented here, we can now gain a better understanding of the role of Nr4a2 ~~_in relation to DA neuron generation, for which this orphan receptor is best known for, by~~ using the advantages of the zebrafish model to visualise and follow these developing cells *in vivo*. It will be particularly useful to characterise its specific role in DA neurons, as the Nr4a2 orphan receptor has already been implicated in DA neurogenesis. The expression of Nr4a2 in non-DA neurons is consistent with other labelling studies across vertebrates and marking all Nr4a2 expressing neurons in this line will allow us to better understand, which differences at which developmental stage contribute to the generation of a DA or non-DA neuron. This may reveal complex patterns of co-expression with other factors or some timing / expression level differences. We can directly assess the role that Nr4a2a and other genes play during fate specification and later differentiation stages. Focusing on other non-DA subtypes expressing Nr4a2a in this transgenic line will be useful for identifying related cell fates that might be reprogrammed into DA neurons.

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Conflict of interest statement

The authors have no conflict of interest.

Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. This project was carried out in the laboratories of WAH & PDC. PJ, JH generated the transgenic line, PJ performed experiments Figure 1 – 7, AW carried out antibody specificity experiments, LG & AW designed and performed experiments Figures 8 – 10. All authors contributed to manuscript preparation.

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Figure Legends

Figure 1. Comparison of *Tg(nr4a2a:eGFP)* expression and *nr4a2a* endogenous *in situ* RNA signal in wholemount zebrafish embryos. Robust *nr4a2a in situ* signal is observed in various central nervous system areas at 48 hpf (A, B), 72 hpf (E, F, F' lateral) and 96 hpf (I, J). At equivalent time, strong *Nr4a2a:eGFP* expression is present in many of the same areas (C, D, G, H, I, J). Additional *eGFP* expression is evident in some areas at 48 hpf (C, D compared to A, B) and more obviously so at 72 hpf (G, H compared to E, F) and 96 hpf (K, L compared to I, J), when *nr4a2a in situ* signal is starting to decrease in many areas. Arrowheads at 96 hpf show large areas of the hindbrain and spinal cord that no longer show RNA expression, but are still labelled with the *Nr4a2a:eGFP* transgene. FB: Forebrain; Ret: Retina; OT: Optic tectum; MB: Midbrain; HB: Hindbrain; SC: Spinal cord. Scale bar in L (A – L) is 100 μ m.

Figure 2. Temporal-spatial expression of *Tg(nr4a2a:eGFP)* transgenic fish line in wholemount zebrafish embryos. Left column shows lateral view, right column shows dorsal view for all time points. Faint *Nr4a2a:eGFP* expression is first observed at 24 hpf and increases in different regions of the central nervous system as developmental time progresses (A – G). The reporter transgene remains during this time, such that any expressing CNS region maintains strong *eGFP* labelling. By 60 hpf strong *eGFP* expression can be observed in distinct retinal neurons (G higher power inset). FB: Forebrain; Ret: Retina; MB: Midbrain; HB: Hindbrain; SC: Spinal cord. Scale bar G (A – G) is 200 μ m, scale bar G inset is 100 μ m.

Figure 3. Co-labelling of *Nr4a2a:eGFP* transgene and endogenous *nr4a2a* mRNA in the retina. Section *in situ* hybridisation of *nr4a2a mRNA* visualised by Fast Red combined with *GFP* immunohistochemistry in our *Tg(nr4a2a:eGFP)* line at 5 dpf. A – A'' Endogenous *nr4a2a* mRNA expression is maintained in a subpopulation of amacrine cells in the INL (red in A, A'').

Nr4a2a:GFP expression at this age is restricted to a subpopulation of amacrine cells in the INL and additionally in some cells in the GCL (green in A', A''). The merged channel (A'') reveals co-labelling of GFP in Fast Red labelled cells as well as additional GFP only expressing cells, that presumably no longer express the endogenous mRNA. **a – a''** High power inset of box shown in A – A''. Co-labelling at single cell resolution can be easier seen at high power, where double labelled cells are indicated by asterisks. **B – B''** High power view of a different section showing the same result, with cells in the INL expressing the endogenous *nr4a2a mRNA* (red) co-labelling with the Nr4a2a:GFP transgene (green) as indicated by asterisks. INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar A (A – A'') is 20 μm , scale bar a (a – a'', B – B'') is 10 μm .

Figure 4. Temporal expression of *nr4a2a mRNA in situ* signal and *Tg(Nr4a2a:eGFP)* transgene in retinal sections of developing zebrafish embryos. **A – G** Endogenous *nr4a2a mRNA* is expressed primarily in developing cells in the inner half of the inner nuclear layer, where mature amacrine interneurons reside. When neural differentiation begins at 28 hpf, there are no *nr4a2a* expressing cells. Expression can be seen robustly starting at 40 hpf (B). As development progresses, the number of cells expressing *nr4a2a mRNA* reduces. **H – N** Nr4a2a:eGFP is also first observed in very few cells at 40 hpf. As development progresses, Nr4a2a:eGFP is also expressed most strongly in the amacrine layer mirroring the mRNA expression. The stable eGFP continues to be expressed longer than the transient mRNA, such that at 120 hpf (N compared to G) there are still more GFP+ cells compared to those expressing endogenous *nr4a2a mRNA*. Additionally Nr4a2a:eGFP expression is also observed in numerous cells of the future ganglion cell layer, where mRNA signal is rarely observed, suggesting either labelling of additional neurons in the transgenic line or labelling of cells express the endogenous mRNA only very transiently. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Scale bar N (A – N) is 50 μm .

Figure 5. Temporal expression of Nr4a2a:eGFP during *live* development within the amacrine lineage. **A – H** Micrographs (2 hourly) from a time-lapse movie of double transgenic *Tg(ptf1a:RFP / nr4a2a:eGFP)* zebrafish embryo. The brightness of the entire figure is adjusted simultaneously, such that individual panels are directly comparable. Ptf1a:RFP expression in developing inhibitory neurons can be observed at 35 hpf. At this time, Nr4a2a:eGFP transgene starts being expressed in Ptf1a:RFP unlabelled ganglion cells (A, B). By 40 hpf (C, D), some of the inner nuclear layer cells start expressing Nr4a2a:eGFP labelled weakly. Expression increases in this layer as development progresses. Ptf1a:RFP labelled horizontal cells that start migrating back apically around 45 hpf (F) do not express Nr4a2a:eGFP. **I** Micrograph of a 45 hpf snapshot of the movie. Magnified inset reveals that Nr4a2a:eGFP expressing cells in the inner nuclear layer co-label with Ptf1a:RFP (white dots). HC: horizontal cells; AC: amacrine cells; GC: ganglion cells. Scale bar H (A – H) is 50 μ m, scale bar I is 50 μ m, scale bar I''' (I' – I''') is 10 μ m.

Figure 6. Expression of Nr4a2a:eGFP and *nr4a2a* mRNA in some neurons in the ganglion cell layer of the retina. **A, B** Expression of transgenic Nr4a2a:eGFP consistently labels cells in the amacrine layer (ACL) and additionally cells in the ganglion cell layer (GCL) as seen in 5 dpf retinal sections. **C – H** Even though rarely observed in retinal sections at 5 dpf, *nr4a2a in situ* labelled cells can be observed in GCL at earlier 48 hpf or 72 hpf in wholemount (C – E) and much more rarely in retinal sections (F – H), suggesting that eGFP in this layer may represent perdurance of the stable transgene in cells that express the endogenous transcript only very transiently. **F' – H'** Higher magnification insets of boxes shown in F – H. Arrowheads indicate *nr4a2a* mRNA labelling detected by *in situ* hybridisation in individual cells in the ganglion cell layer. Scale bar B (A, B) is 50 μ m, scale bar E (C – E) is 50 μ m, scale bar H (F – H) is 50 μ m, scale bar H' (F' – H') is 10 μ m.

Figure 7. *Live* expression of Nr4a2a:eGFP exclusively within the Atonal 7 lineage in all retinal layers. **A** Micrograph of a 45 hpf snapshot of a movie of double transgenic *Tg(ato7:RFP /*

nr4a2a:eGFP) zebrafish embryo. Nr4a2a:eGFP expression in both the ganglion cell layer (GCL) and amacrine cell layer (ACL) in the inner nuclear layer co-label with the membrane Atoh7:RFP transgene. Magnified inset shows co-labelled cells in the GCL and (white dots). Scale bar A is 50 μm , scale bar A''' is 10 μm .

Figure 8. Co-labelling of *Tg(nr4a2a:eGFP)* and tyrosine hydroxylase immunoreactivity in retinal sections of 5 dpf zebrafish embryos. **A – C** DAC in the retina labelled with tyrosine hydroxylase immunohistochemistry were also marked by a small population of the Nr4a2a:GFP labelled amacrine cells (asterisks indicate double labelled cells in the insets showing boxed area at higher magnification). Scale bar C (A – C) is 20 μm , scale bar C' (A' – C''') is 10 μm .

Figure 9. Immunohistochemical co-labelling of *Tg(nr4a2a:eGFP)* with non-dopaminergic amacrine subpopulation markers in retinal sections of 5 dpf zebrafish embryos.

A – H Micrographs showing retinal overview (left panel) and higher power view of individual as well as combined red and green channels of boxed regions for each of the amacrine subtype markers used. **A** Some of the GABAergic amacrine interneuron subpopulations were co-labelled with cells expressing varying degrees of Nr4a2a:eGFP (white dots), though GABAergic amacrine cells that did not express any Nr4a2a:eGFP were also observed (white asterisks). **B – H** For the vast majority of amacrine subtype or subpopulation markers, there was very little co-labelling with Nr4a2a:eGFP (white dots), with the majority of immunohistochemically labelled cells not expressing any detectable Nr4a2a:eGFP (white asterisks). INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar in left panel H (A – H) is 50 μm , scale bar inset H (A – H) is 10 μm .

Figure 10. Co-labelling of amacrine subtype markers with three distinct intensities of *Tg(nr4a2:eGFP)* transgene labelling. **A** Micrograph of retinal sections from zebrafish at 5 days post

fertilisation (dpf) reveal different intensity of transgene staining (1 – weak, 2 medium, 3 strong GFP in higher power inset A' of boxed region in A). **B** Graph showing subjective categorization (1 – 3) and corresponding intensity measurement. The transgene intensity differences quantitatively (0 – 255) fall into three distinct populations (three peaks with dips in-between), which match subjective classification well. The small overlap that can be observed in the graph is due to variations between samples. Categorization within individual images never showed any such overlap. **C** Using the three intensities, the co-localisation of Nr4a2a with various amacrine subtype markers was quantified. As expected Nr4a2a completely co-labelled with tyrosine hydroxylase immunoreactive dopaminergic neurons, which primarily expressed a medium level of Nr4a2a:eGFP transgene. Nr4a2a:eGFP was expressed to varying degrees in the multiple GABAergic amacrine subpopulations, but showed only very minimal co-expression with any other established amacrine subtype marker including the four calcium binding proteins parvalbumin (PV), calretinin, secretagogin (SCGN) and calbindin, as well as choline acetyltransferase (ChAT), neuropeptide Y (NY) and serotonergic (5HT) labelled amacrine cells. n = number of cells analysed for each immunohistochemical marker. INL: inner nuclear layer; GCL: ganglion cell layer. Scale bar A is 50 μm , scale bar inset A is 10 μm .

In vivo expression of Nurr1 / Nr4a2a in developing retinal amacrine subtypes
in zebrafish *Tg(nr4a2a:eGFP)* transgenics

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Abstract

The Nuclear receptor subfamily 4 group A member 2 (Nr4a2) is crucial for the formation or maintenance of dopaminergic neurons in the central nervous system including the retina, where dopaminergic amacrine cells contribute to visual function. Little is known about which cells express Nr4a2 at which developmental stage. Furthermore, whether Nr4a2 functions in combination with other genes is poorly understood. Thus, we generated a novel transgenic to visualise Nr4a2 expression *in vivo* during zebrafish retinogenesis. A 4.1 kb fragment of the *nr4a2a* promoter was used to drive green fluorescent protein expression in this Tg(*nr4a2a:eGFP*) line. *In situ* hybridisation showed that transgene expression follows endogenous RNA expression at a cellular level. Temporal expression and lineages were quantified using *in vivo* time-lapse imaging in embryos. Nr4a2 expressing retinal subtypes were characterised immunohistochemically. Nr4a2a:eGFP labelled multiple neuron subtypes including 24.5% of all amacrine interneurons. Nr4a2a:eGFP labels all tyrosine hydroxylase labelled dopaminergic amacrine cells, and other non-dopaminergic GABAergic amacrine populations. Nr4a2a:eGFP is confined to a specific progenitor lineage identified by sequential expression of the bhlh transcription factor Atonal7 (Atoh7) and Pancreas transcription factor 1a (Ptf1a), and labels post-mitotic post-migratory amacrine cells. Thus, developmental Nr4a2a expression indicates a role during late differentiation of specific amacrine interneurons. Tg(*nr4a2a:eGFP*) is an early marker of distinct neurons including dopaminergic amacrine cells. It can be utilised to assess consequences of gene manipulations and understand whether Nr4a2 only carries out its role in the presence of specific co-expressed genes. This will allow Nr4a2 use to be refined for regenerative approaches.

Background

Within the retina the five main types of neurons can be subdivided into many subtypes that form distinct circuits to analyse different aspects of vision in parallel. The most diverse population are amacrine interneurons, which synapse onto and modulate signals between bipolar and ganglion cells in the inner plexiform layer of the retina (Masland, 1988). Dopaminergic interplexiform or amacrine cells are present in all vertebrate retinas (reviewed in Witkovsky, 2004; reviewed in Popova, 2014). While these retinal subtypes form specific connections including GABAergic synapses (releasing the neurotransmitter gamma-aminobutyric acid) onto various distinct post-synaptic neurons (Contini and Raviola, 2003; Volgyi et al., 2014; Debertin et al., 2015), dopamine itself acts as a paracrine neuromodulator, which can affect all retinal neurons via different G-protein coupled dopamine receptors (Contini and Raviola, 2003; reviewed in Popova, 2014; Hirasawa et al., 2015). Dopamine synthesis and release is modulated by light levels through cone and rod photoreceptors and melanopsin ganglion cells, as well as circadian rhythms (Sakamoto et al., 2005; Cameron et al., 2009; Van Hook et al., 2012; Zhang et al., 2012; reviewed in Popova, 2014; Vuong et al., 2015; Qiao et al., 2016). Although different experimental setups and species have yielded different data on its precise action, dopamine certainly affects visual circuits involved in light adaptation, colour discrimination, contrast sensitivity, acuity and circadian rhythms, as well as playing important trophic roles including eye growth (reviewed in Witkovsky, 2004; Jackson et al., 2012; Feldkaemper and Schaeffel, 2013; Ventura et al., 2013; Kim et al., 2014; Nebbioso et al., 2014; reviewed in Popova, 2014).

The proper generation of DAC like their DA counterparts across other brain areas is crucial for normal neurological functioning. The orphan nuclear hormone receptor Nurr1 / Nr4a2 first identified in 1992 (Law et al., 1992) plays an important role in the development of DA neurons across the central nervous system. Nr4a2 has been of particular interest, as its loss affects terminal differentiation and survival of DA neurons in the substantia nigra (Zetterstrom et al., 1997;

Saucedo-Cardenas et al., 1998; Le et al., 1999; reviewed in Smidt and Burbach, 2009) and as it has been linked to Parkinson's disorder (Le et al., 2003; Liu et al., 2012a; Liu et al., 2012b).

Key differentiation factors including dopamine transporter and tyrosine hydroxylase (TH) are regulated through Nr4a2 acting synergistically with factors including Pitx3 and Foxa2 (reviewed in Arenas, 2005; Martinat et al., 2006; Jacobs et al., 2009; reviewed in Smidt and Burbach, 2009; Lee et al., 2010). These studies suggest that co-operation of different transcription factors may explain, at least in part, how subtype specification is achieved, even though individual factors themselves are expressed more broadly (Martinat et al., 2006). Identified pathways that regulate Nr4a2 include the Wnt signalling pathway through β -catenin binding of Nr4a2 (reviewed in Smidt and Burbach, 2009) and retinoid signalling (Volakakis et al., 2009).

In the zebrafish, two paralogues (a and b) with overlapping expression patterns have been identified, with Nr4a2a being more homologous to the Nr4a2 in higher vertebrates (Blin et al., 2008). *Nr4a2a* knockdown in zebrafish also showed the more drastic loss of DA phenotype in different CNS regions, equivalent to the phenotype obtained from knocking down both paralogues simultaneously, whilst *nr4a2b* knockdown did not display as severe a phenotype (Filippi et al., 2007). Thus, these paralogues act non-redundantly, with the *nr4a2a* paralogue most closely mirroring the described function of Nr4a2 in mammalian vertebrates.

In the vertebrate retina, Nr4a2 is expressed in a subpopulation of amacrine cells, including the DA subtypes (Filippi et al., 2007) and other GABAergic amacrine subtypes (Jiang and Xiang, 2009). Dominant-negative forms of Nr4a2 in the mouse and morpholino knockdown of Nr4a2a, but not Nr4a2b in the zebrafish results in an absence of DAC (Filippi et al., 2007; Jiang and Xiang, 2009). Misexpression in mouse has been shown to promote GABAergic amacrine differentiation. These studies suggest that in the retina, Nr4a2 may have a general role in the development of GABAergic including DAC.

In order to investigate further the role and expression profile of Nr4a2a during development of these retinal cell types, we generated a transgenic line that faithfully recapitulates the expression

of endogenous *nr4a2a* RNA. By using enhanced green fluorescent reporter protein (eGFP), we are able to follow cells as they continue to mature and differentiate, assessing their final fate in terms of distribution and subtype composition, even though these cells may express *nr4a2a* only transiently during development. We confirmed that many retinal neurons belonging to multiple subtypes, which include all DA amacrine cells, express Nr4a2a. The genetic context (lineage) and temporal expression of Nr4a2a was characterised to elucidate during which stage of neurogenesis Nr4a2a function is most important. Our data shows that Nr4a2 expression in amacrine cells occurs only for a brief period during early post-mitotic differentiation, and that distinct AC subtypes express Nr4a2a during their development.

Methods

Animals

Zebrafish were maintained and bred at 26.5°C, and embryos of either gender used for experiments were raised at 28.5°C and staged as previously described (Kimmel et al., 1995) in hours postfertilisation (hpf). All procedures were carried out according to the provisions of the Australian National Health and Medical Research Council code of practice for the care and use of animals and were approved by the institutional ethics committee at the University of Cambridge, Monash University and University of Melbourne.

Transgenic construct cloning

A 4094 bp fragment of the zebrafish *nr4a2a* promoter region was PCR amplified from the BAC clone RP71-49K16 (Children's Hospital Oakland Research Institute, Oakland, USA) using advantage taq (Contech, 639201). Forward primer: 5'-GGGGACAACCTTTGTATA GAAAAGTTGCCCCCTCTATCCCTGTCACACA-3' and reverse primer: 5'-GGGACTGCTTTTTTGTACAAAGCTGGCTGCAATTAAACAAA-3'. The full *nr4a2a*:eGFPpA construct was generated using the tol2kit. The PCR product was recombined into the pDONRP4-P1R gateway entry clone, sequence verified and recombined via LR reaction with gateway entry clones pDEST vector containing tol2. The pME-eGFP and p3E-polyA entry clones were kindly provided by Dr K Kwan and Professor CB. Chien (Kwan et al., 2007).

Transgenic zebrafish generation

The transgene construct (50 ng / μ l) was injected into the cell of one-cell stage WT zebrafish embryos together with (50 ng / μ l) Tol2 transposase mRNA (1 – 2 nl / embryo). Transposase

mRNA was synthesised using mMessage mMACHINE (Amersham). Screening of transgenic zebrafish throughout multiple outcrossed generations is described in the results section.

Immunohistochemistry

Zebrafish embryos and larvae were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline pH 7.4 (PBS) at room temperature (RT) for 3 hrs or overnight at 4°C. Zebrafish were rinsed, cryoprotected in 30% sucrose, embedded in OCT (Tissuetek) and cryosectioned at 14 µm thickness. All immunohistochemistry steps were performed at RT.

Sections were incubated in blocking buffer (5% foetal calf serum, 1% bovine serum albumin, 0.5% Triton X-100 in PBS) for 30 min. Sections were incubated in primary antibodies diluted in blocking buffer overnight. Primary antibodies used in this study are listed in Table 1. Sections were rinsed in PBS and incubated in secondary antibodies for 1 hour at RT. Secondary antibodies diluted in blocking buffer were goat or donkey anti-mouse, anti-rabbit or anti-goat IgG conjugated to Alexa 488 or 546 fluorophores (1:500 dilution, Molecular Probes). After further rinses, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and sections were coverslipped in Mowiol.

Antibody characterisation

The mouse anti-tyrosine hydroxylase antibody (RRID: AB_2201528) is used here to specifically label dopaminergic neurons. The antibody is raised against a rate-limiting enzyme during dopamine production. This commercial antibody was raised against TH purified from rat PC12 cells. It recognises an epitope outside of the regulatory N-terminus, and a 62 kDa band in immunoblots of zebrafish brain (Uyttbroek et al., 2010). In zebrafish CNS this antibody exclusively labels TH1 as shown by co-localisation with *th1*, but not *th2* *in situ* hybridisation (Chen et al., 2009).

Additionally, immunolabelling with this antibody is abolished following morpholino mediated knockdown specifically of Th1 in embryonic zebrafish CNS (Kuscha et al., 2012).

The rabbit anti-gamma-aminobutyric acid antibody (RRID: AB_477652) was used to label a large subpopulation of amacrine cells, which express the GABA neurotransmitter. The antibody specifically recognises GABA in a dot blot assay (manufacturer's datasheet, species not mentioned). The structure of GABA is identical in all vertebrate species. Within the zebrafish retina, the staining pattern obtained using this GABA antibody is comparable with that obtained using antibodies against GAD65 and GAD67, the glutamic acid decarboxylates, which are the enzymes that convert glutamate to GABA (Connaughton et al., 1999).

The rabbit anti-calbindin D-28K antibody (RRID:AB_213554) specifically recognises the expected 28 kDa band in Western blots in zebrafish brain homogenate. Calbindin immunoreactivity in zebrafish is abolished when the primary antibody is preabsorbed with recombinant rat calbindin (Uytenbroek et al., 2010).

The rabbit anti-calretinin antibody (RRID: AB_2068506) specifically recognises a 28kDa Western blot band in zebrafish brain homogenate (Uytenbroek et al., 2010).

The goat anti-choline acetyl transferase antibody (RRID: AB_2079751) recognises a specific 70 kDa band on Western blots of mouse brain lysate (manufacturer's datasheet). In zebrafish, immunoreactivity is abolished when the antibody is preabsorbed with recombinant rat ChAT (Olsson et al., 2008).

The chicken anti-green fluorescent protein antibody (RRID: AB_2534023) was directly raised against GFP isolated from jellyfish (*Aequorea victoria*) and the IgY fraction was purified by affinity purification (manufacturer's datasheet). The antibody recognised purified GFP consisting of 238 amino acids at 27 kDa (Hamamoto et al., 2016). The GFP transgene is unrelated to any zebrafish proteins and staining of GFP in non-transgenic zebrafish results in no immunoreactivity (data not shown).

The rabbit anti-neuropeptide Y antibody (RRID: AB_572253) specifically stains NY in the rat central nervous system, which is blocked by preabsorption (manufacturer's datasheet). NY is a small 36 amino acid neurotransmitter that is highly conserved across all vertebrates, with zebrafish NY showing 89% homology to the human NY (Soderberg et al., 2000). In goldfish, preabsorption of this antibody with rat NY abolishes immunoreactivity in the brain and pituitary as shown by immunoblotting, enzyme immunoassay and radioassay (Matsuda et al., 2009).

The mouse anti-parvalbumin antibody (RRID: AB_2174013) specifically stains Ca²⁺ bound forms of parvalbumin and recognises a 12 kDa protein in Western blots of mouse brain lysate (manufacturer's datasheet) and whole zebrafish homogenate (our data not shown).

The rabbit anti-secretagogin antibody (RRID: AB_2034060) used shows identical staining in zebrafish to a commercial sheep anti-secretagogin antibody not used for analysis in this study (data not shown, RRID: AB_2034062). In mammalian species both of these antibodies label the same cells as a non-commercial secretagogin antibody that recognise the 32 kDa predicted band in mouse retina and cerebellum (Puthussery et al., 2010; Weltzien et al., 2014).

The rabbit anti-serotonin antibody (RRID: AB_477522) reacts with serotonin-containing fibers in rat brain and specific staining is inhibited by preabsorption with serotonin or serotonin-BSA (manufacturer's datasheet). 5-HT is identical in all vertebrate species (Olsson et al., 2008). Preabsorption with 5HT- BSA conjugate completely abolishes zebrafish staining (Uytenbroek et al., 2010).

Wholemout and section in situ hybridisation

In situ RNA hybridisation was performed following standard protocols for wholemount embryos (Thisse and Thisse, 2008) or cryostat sections (Butler et al., 2001). Constructs for *nr4a2a* RNA probes were kindly provided by Professor Driever. For antisense RNA probe, DNA was linearised with XhoI and transcribed with SP6 RNA polymerase.

Embryos were fixed in 4% PFA overnight, rinsed and processed as wholemounts or cryoprotected in 30% sucrose, embedded in OCT and cryostat sectioned at 20 μ m thickness.

Wholemount embryos were dehydrated serially into 100% methanol, rehydrated into PBST (0.1% Tween 20 in PBS), digested in proteinase K (0.25 – 25 μ g/mL depending on age) for 25 minutes, postfixed in 4% PFA, prehybridised at 65°C for 2 – 6 hours and hybridised with RNA probe overnight at 65°C. Unbound probe was removed by 65°C serial dilution from 50% formamide / 2X SSC through 2X SSC, then 0.2X SSC and finally into PBST at room temperature. Embryos were blocked (2% sheep serum, 5% bovine serum albumin in PBST) incubated in preabsorbed anti-Dig Fab fragment antibody (1:100) overnight, rinsed, equilibrated in staining buffer (100 mM NaCl, 0.1% Tween 20 in 0.1M Tris pH 9.5) and stained with NBT/BCIP (Roche Diagnostics 11681451001). After postfixation with 4% PFA, embryos were equilibrated into 100% glycerol and imaged.

For section *in situ* hybridisation, sections were postfixed with 4% PFA, rinsed in 2x SSPE, incubated 0.1M triethanolamine without and then with 0.25% acetic anhydride, rinsed, prehybridised at 60°C for 2 – 5 hours followed by probe hybridisation overnight at 60°C.

Sections were rinsed in 2x SSPE at RT, 20 μ g/ml RNase A in 4x SSPE at 37°C for 30 minutes and 50% formamide in 2x SSPE at 60°C for 45 minutes. After rinses in 2x SSPE at and buffer 1 (150 mM NaCl in 0.1M Tris pH 7.5) at room temperature, sections were blocked in 0.5% blocking reagent (Roche) in buffer1 for 2 hours and incubated in preabsorbed anti-Dig Fab fragment (1:100) for 1.5 hours. Antibody was rinsed off with buffer 1, equilibrated in staining buffer (100 mM NaCl in 0.1M Tris pH 9.5) and signal was visualised with NBT/BCIP or Fast Red (Sigma, F4648) followed by coverslipping in Mowiol.

Imaging of fixed and live embryos

Wholemount fixed transgenic embryos were imaged on the SZX2-ILLB stereo microscope (Olympus) using cellSense Standard software (Olympus). Wholemount *in situ* hybridisation stained embryos were imaged on a BX51 microscope with dotSlide software (Olympus). Sections were photographed at the Z1 Axioscope using Axiovision software (Zeiss) using the Apotome. Images were processed in Adobe Photoshop (brightness and contrast adjustments) and figure panels combined in Adobe Illustrator.

For *live* time-lapse imaging, embryos were treated with 0.002% phenylthiourea (PTU) from 24 hpf onwards to delay eye pigment formation, and embedded in 1% low melt agarose / 0.002% PTU / 0.04% tricaine methane sulfonate in E3 embryo medium (Jusuf et al., 2013). Imaging was performed with a confocal system (Zeiss LSM5/710 meta) using a water immersion 20X objective and 488 nm argon (GFP) and 560 nm laser lines (RFP). Optical sections (120 – 150 μm) of 3 μm thickness were taken through the eye every 30 min for 24 hours. Image data were acquired using Zeiss ZEN software and processed using Imaris and Fiji. Brightness and contrast were adjusted in Adobe Photoshop.

Analysis

Numbers of embryos / cells analysed are indicated in the relevant results section.

Quantification of the percentage of retinal amacrine cells expressing Nr4a2a:eGFP was performed using rectangular regions of interest (ROIs) that were 50 μm wide and encompassed ~ 40 amacrine cells per ROI.

The brightness intensity of transgene expression in individual cells was quantified in Fiji (n = 251 cells, n = 6 embryos). A small rectangular ROI within the centre of each cell (with dimensions about half of the cell body) was chosen and intensity measured using the “Colour histogram”

analysis tool (0 – 255). This measure is independent of the size of the ROI, provided the ROI remains within the boundaries of the cell.

Co-labelling of Nr4a2a:eGFP expression in retinal amacrine subtypes / subpopulations were quantified in sections of 5 dpf retinas. The number of cells analysed for each are indicated in the relevant section. For markers that label a relatively dense population of cells, images were randomly chosen by arbitrarily choosing a section with or close to the optic nerve section, though the co-labelling pattern should be the same across the entire retina. For markers that label a relatively small population of cells, the number of embryos sampled was increased to ensure sufficient cell number for analysis and every section containing a labelled cell was imaged and included in analysis. Analysis was conducted relative to the marker, i.e. how many immunolabelled cells expressed Nr4a2a:eGFP and at which intensity.

Results

Tg(nr4a2a:eGFP) zebrafish line generation

Injected zebrafish embryos screened for mosaic GFP expression at 2 – 5 days postfertilisation (dpf) resulted in approximately 800 embryos grown to adulthood. Offspring (n = ~100 – 500) of 27 putative founders were screened for pattern of eGFP expression at 2, 3, 4 and 5 days postfertilisation (dpf). We identified germ-line transmission in 17 of 27 screened founder fish. Nine of these founders showed eGFP reporter expression in the expected central nervous system (CNS) regions and F1 families were raised of these. Each of the F1 fish was outcrossed with WT and F2 offspring were screened for Nr4a2a:eGFP expression. We identified five independent outcrossed F1 fish from three original injected F0 fish (ID14, 26 and 27) that showed the expected pattern of Nr4a2a:eGFP expression in the retina. These five founders showed comparable and stable expression patterns. Outcrossed offspring raised to the F3 or F4 generation were used for further characterisation including time-series of Nr4a2a:eGFP expression in wholemount *live* larvae and comparison to *in situ* RNA signal.

Temporal transgene expression follows endogenous RNA in situ pattern

We characterised the expression pattern of the eGFP reporter transgene at key developmental ages, at which strong endogenous *nr4a2a* RNA *in situ* hybridisation signal was previously described (Filippi et al., 2007). At 48 hpf, strong *nr4a2a* promoter driven eGFP expression was observed in the head and spinal cord (Figure 1A – D).

At this age, the *nr4a2a* RNA signal is expressed in the different CNS regions including the retina and spinal cord (Figure 1A-D) as well as ventral telencephalon, diencephalon, tegmentum and medulla oblongata consistent with previous descriptions (Filippi et al., 2007; Blin et al., 2008). By 72 hpf and 96 hpf the endogenous expression of *nr4a2a* RNA is more restricted in many regions including spinal cord and hindbrain (black arrowheads, Figure 1), in which Nr4a2a might be

particularly important only during developmental stages of these structures. The transgenic line shows strong eGFP expression throughout the CNS and is maintained in regions that have now started downregulating endogenous expression (white arrowheads, Figure 1), due to the perdurance of the eGFP transgene with half-life of > 1 day (Andersen et al., 1998; Corish and Tyler-Smith, 1999) (Figure 1E – L).

A detailed time series was conducted to follow the onset of the eGFP signal during these early stages of CNS development. Strong reporter protein expression could be first visualised at 24 hpf and 30 hpf in the telencephalon (Figure 2A, B). Transgene eGFP was rapidly upregulated across the CNS between 36 hpf – 48 hpf, such as the midbrain and the hindbrain, and continued in spinal cord cells (Figure 2C – E). Between 48 – 60 hpf, eGFP expression was relatively stable and remained strong in all areas (Figure 2F, G). At this age, retinal expression of Nr4a2a:eGFP can be seen (higher power inset Figure 2G). Thus, cells that turn on Nr4a2a:eGFP in the expected CNS areas maintain stable eGFP expression.

Nr4a2a:eGFP labelled cells co-label with endogenous nr4a2a RNA in the retina

Similarly, the expression of *nr4a2a* RNA *in situ* signal and Nr4a2a:eGFP transgene expression was compared in retinal sections of 5 dpf embryos, where individual cells could be identified (Figure 3A – B''). By this stage the central retina has been fully developed and functional for at least 24 hours. In the central retina, co-localisation with the eGFP transgene signal could be seen for 82% of cells also labelled with *nr4a2a* RNA *in situ* signal (n = 155 cells, asterisks in inserts, Figure 3a – a'', B – B''). The immunohistochemical recovery of the GFP signal is variable after *in situ* hybridisation resulting in fewer and generally weaker GFP signal after such procedures (Figure 3). By 5 dpf, there were additional neurons positive only for Nr4a2a:eGFP in these regions. This is consistent with expression of our transgenes in cells that still express *nr4a2a* mRNA additional to those that have meanwhile switched off *nr4a2a* mRNA expression. The eGFP was

specifically chosen to provide a strong label allowing us to follow cells in which endogenous mRNA as well as protein may or may no longer be expressed in at the time of analysis.

Nr4a2a:eGFP expression recapitulates temporal and spatial pattern of the endogenous RNA expression

A comparative time-series of endogenous *nr4a2a* RNA expression by *in situ* hybridisation and *Tg(nr4a2a:eGFP)* transgene expression was conducted in the retina. At 28 hpf when the first progenitors exit the cell cycle to start generating the first born retinal neurons (retinal ganglion cells), there is neither expression of endogenous *nr4a2a* RNA nor GFP transgene (Figure 4A, H). Amacrine interneurons first start differentiating at 35 hpf as they turn on expression of Pancreas transcription factor 1 a (Ptf1a) after multipotent progenitors undergo their final apical division and start migrating down to the future amacrine cell layer (ACL) in the inner nuclear layer (INL), which forms in the middle of the retina (Jusuf et al., 2009). At 40 hpf, we first observe cytoplasmic *nr4a2a* RNA expression in the centre of the retina, where the future amacrine layer is forming (Figure 4B). No labelled nuclei are ever observed more apically, suggesting that *nr4a2a* expression is activated only once cells that have migrated to their final location. At this time-point the *Nr4a2a:eGFP* transgene is only detected very rarely in a few cells closest to the lens (Figure 4I). By 48 hpf, when endogenous RNA expression and transgene expression is strong in the ACL (Figure 4C), eGFP positive cells are additionally observed in the ganglion cell layer (Figure 4J). At 60 hpf the endogenous RNA *in situ* signal starts to be downregulated in the ACL closest to the inner plexiform layer (Figure 4D). Finally, endogenous *nr4a2a* RNA expression becomes further restricted to only a few amacrine cells by 120 hpf (Figure 4E – G). The strongest *Nr4a2a:eGFP* expression at these time-points is similarly in the ACL (Figure 4L – N). The subsequent loss of endogenous mRNA staining suggests that *Nr4a2a* may be particularly important during a specific period of development, whereas the maintenance of *in situ* signal for *nr4a2a* mRNA in a few specific cells might point towards a secondary role in maintenance or survival of a subset of these neurons. The density and distribution of the cells expressing the endogenous *nr4a2a* mRNA at 120 hpf (Figure

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4G) is similar to the pattern observed with antibody staining for dopaminergic neurons at this stage (Figure 6).

In the inner nuclear layer Nr4a2a:eGFP is expressed exclusively in post-mitotic and post-migratory amacrine interneurons

Using the Tg(*nr4a2a:eGFP*) line, we performed *in vivo* time-lapse imaging experiments to correlate the timing of transgene expression to the different stages of neural differentiation. Ptf1a is a transcription factor that is expressed transiently during the development of all amacrine cells (Jusuf et al., 2011), of which DA neurons are a subtype. Using a double transgenic line additionally expressing RFP under the *ptf1a* promoter, we can visualise inhibitory neurons just after they undergo their last progenitor division apically and migrate to the forming amacrine layer (Figure 5A - H). As previously described, Ptf1a:RFP can be first detected around 35 hpf, when amacrine cells start to differentiate (Jusuf and Harris, 2009). At this time, the Nr4a2a:eGFP transgene is just starting to be detected faintly in the ganglion cell layer in Ptf1a unlabelled ganglion cells (Figure 5A). By 41 hpf, Nr4a2a:eGFP transgene can be visualised in the ACL (Figure 5D), suggesting it is first expressed about 5 – 6 hours after Ptf1a expression, and consistent with our fixed samples, only in cells that have already finished migrating and forming the future amacrine layer. The vast majority of cells that have just started expressing Nr4a2a:eGFP in the INL are expressing Ptf1a:RFP (93%, n = 358 cells, 14 images from 5 time-lapse movies), consistent with specification as amacrine interneurons (Figure 5I, white dots mark cells co-expressing Nr4a2a:eGFP and Ptf1a:RFP). Ptf1a:RFP labelled horizontal cells that start migrating back apically around 45 hpf never express Nr4a2a:eGFP (Figure 5F - H). These time-lapse experiments identify the expression of Nr4a2 in post-mitotic post-migratory neurons, indicating that its role is confined to later stages of neurogenesis that might include subtype fate specification and other terminal differentiation processes. Additionally, Nr4a2a is expressed in a specific subpopulation of Ptf1a expressing neurons that includes some amacrine, but not horizontal cells.

Post-mitotic Nr4a2a:eGFP expressing retinal neurons in all layers arise exclusively from the Atonal 7 lineage

As seen in our fixed tissue (Figure 4I – N) and the time-lapse imaging (Figure 5A – H), we unexpectedly observed Nr4a2a:eGFP labelling in the ganglion cell layer, which has not been described for zebrafish retina previously. The intensity of the transgene, which might be a qualitative indicator of promoter activation is variable in cells within this layer, though usually weaker than the strongest expressing cells in the ACL, once they upregulate their expression (Figure 4L – M). Although most of the endogenous RNA *in situ* signal was exclusively observed in the forming ACL in the INL, we did come across a few examples of RNA expressing cells in the GCL in both in wholemount *in situ* preparations (arrows in Figure 6C – E) as well as in sections processed for *in situ* hybridisation (arrowhead in high magnification insets (boxed) in Figure 6F – G). This would be consistent with very transient (and possibly weak) expression of *nr4a2a* mRNA within some of these cells, which remain labelled by our stable strong eGFP expression within this layer.

We used the Tg(*atoh7:RFP*) line to characterise the extent to which Nr4a2a:eGFP positive cells were related to the progenitor lineage marked by the transcription factor Atoh7, which is upregulated to specify ganglion cell fates and downregulated after very transient expression in cells destined to give rise to photoreceptors, horizontal cells and a subpopulation of amacrine cells. Atoh7:RFP is first detected at 28 hpf, just prior to the terminal mitosis of the first born ganglion cells (Poggi et al., 2005). The first Nr4a2a:eGFP positive cells are seen 7 hours later and co-label with the Atoh7:RFP transgene in the ganglion cell layer (Figure 7). Subsequent expression of Nr4a2a:eGFP in the inner nuclear layer where amacrine cells are found also co-labels with Atoh7:RFP (total 98%, n = 646 cells, 19 images from 7 time-lapse movies). Thus Nr4a2 expression in any of the retinal layers only occurs in cells from the Atoh7 lineage. Taken together, these time-lapse experiments identify Nr4a2 expression in a specific subpopulation of retinal neurons, namely those that arise from a neural lineage characterised by sequential expression of Atoh7 and Ptfla..

Dopaminergic neurons within the retina express Nr4a2a:eGFP

Since Nr4a2 has been implicated in generating DA neurons, we first compared the expression of tyrosine hydroxylase protein against our eGFP transgene using immunohistochemistry to ascertain whether the lineage specific subpopulation of retinal neurons include the DAC. Co-staining of tyrosine hydroxylase with *nr4a2a in situ* RNA signal in previous studies already revealed some, but not complete co-labelling (Filippi et al., 2007; Blin et al., 2008). With the perdurant GFP in our transgenic line, we can now assess whether these cells truly never expressed Nr4a2 or only transiently expressed *nr4a2a* RNA.

Tyrosine hydroxylase immunoreactive cells co-labelled with our eGFP transgene in the retina (100%, n = 88 cells from 39 embryos), though unexpectedly not necessarily at the highest transgene intensity level (only 3% expressed the transgene at a high level compared to 53% weakly and 43% at medium brightness, Figure 8A – C, Figure 10C), suggesting that these are not the cells that generate the most Nr4a2a. Nonetheless, these results show that DAC interneurons in the retina arise from cells that express Nr4a2a during their development.

Nr4a2a:eGFP expression in distinct non-dopaminergic retinal amacrine cells

Nr4a2 is expressed in more retinal neurons that are not labelled by dopaminergic markers such as tyrosine hydroxylase. Within the amacrine cell half of the INL (distinguishable by weaker DAPI labelling compared to the more intensely labelled bipolar cell half), Nr4a2a:eGFP is expressed in 24.5% of amacrine cells (n = 509 from 13 zebrafish). Using our transgenic line in combination with established amacrine subtypes or subpopulation (possibly multiple subtypes) markers we further characterised, if Nr4a2a was expressed in specific amacrine subpopulations.

Conserved with reports in other vertebrates, in the zebrafish retina, Nr4a2 is expressed in the majority of GABAergic amacrine subtype (64%, n = 1351 cells from 12 zebrafish), which include

DACs. The intensity of the transgene varied widely, with 18% of GABAergic cells expressing Nr4a2a:eGFP strongly, 24% at medium levels and 22% at weak levels. However, not all GABAergic amacrine cells show Nr4a2 expression (36% did not co-localise), suggesting it is not necessary for the global GABAergic neurotransmitter phenotype (Figures 9A).

In contrast, all Nr4a2a:eGFP expressing retinal cells also expressed GABA. Using other amacrine subtypes markers Nr4a2 was distinctly expressed in amacrine population that primarily did not co-label with any of these markers (asterisks Figure 9B – H). This included antibodies against the calcium binding proteins calretinin (16% co-localisation, $n = 1322$ cells from 15 zebrafish), calbindin (10% co-localisation, $n = 598$ cells from 17 zebrafish) and parvalbumin (25% co-localisation in the INL, $n = 545$ cells from 17 zebrafish, 4% co-localisation in the GCL, $n = 724$ cells from 11 zebrafish), as well as subtype specific markers against secretagogin (22% co-localisation, $n = 589$ cells from 17 zebrafish), choline acetyltransferase (22%, $n = 232$ cells from 6 zebrafish), neuropeptide Y (9% co-localisation, $n = 77$ cells from 30 zebrafish) or serotonin (4% co-localisation, $n = 200$ cells from 14 zebrafish).

Because the transgenic line showed differences in transgene brightness intensities, correlation of subjective categorization with quantitative brightness measures was performed (Figure 10A, B). Cells were assigned into one of four categories: No co-label or co-labelling with weak, medium or strong GFP expression (Figure 10). The brightness of each cell was then quantified (Fiji) and plotted. Three relatively distinct populations with separate peaks could be identified, which corresponded well to subjective categorisation. Brighter labelling can be due to the Nr4a2 promoter either being activated more strongly or for a longer period, both of which could lead to an increase in the number of GFP (and presumably Nr4a2) proteins being generated. Subsequent co-labelling with amacrine markers such as those from Figures 8 and 9 were analysed according to such categories (Figure 10C, $n =$ number of cells analysed for each immunohistochemical marker). This quantification confirmed that TH⁺ DACs all express Nr4a2a (though to varying degree, with the majority expressing Nr4a2 at medium levels). Secondly,

GABAergic amacrine cells (which account for about half of the zebrafish amacrine subtypes) showed substantial co-labelling with Nr4a2a:eGFP, though about a third did not show any detectable transgene labelling. Whether the different Nr4a2a:eGFP intensities correspond to distinct GABAergic subtypes within the GABAergic group could not be determined with available markers. Surprisingly none of the other markers showed much Nr4a2a:eGFP expression. This confirms that Nr4a2 is not randomly expressed to the same proportion within every amacrine subtype but selectively expressed in distinct amacrine subtypes, namely those that are not labelled with our available markers.

Discussion

The correct development of different neuron subtypes is crucial for the formation of neural circuits, each carrying out a distinct function. This is particularly well characterised in the vertebrate retina, where distinct subtypes of the five main types of neurons form specific synaptic circuits that analyse discrete sub-modalities of vision such as colour. Across the CNS most genetic and neurodegenerative disorders affect only specific subtypes. Thus, an understanding of the lineages and gene networks that generate different types of neurons during development forms the basis of understanding genetic neural disorders and direct approaches to recapitulate such developmental processes towards regenerating lost subtypes in the adult CNS. Here, we generated a novel transgenic tool to dissect out the genetic pathways that give rise to particularly (but not exclusively) the dopaminergic neuron subtypes, which include a dopaminergic amacrine interneuron subtype in all vertebrate retinas. Dopaminergic neurons are crucial for a number of catecholamine pathways, which when disrupted are linked to disorders such as Parkinson's or schizophrenia (Jankovic et al., 2005). Within the retina, dopamine released from interplexiform and amacrine cells acts as a paracrine neuromodulator and contributes to light adaptation and circadian rhythms, colour and contrast discrimination and visual acuity due to the role of dopaminergic retinal amacrine cells in this visual sub-modality (Witkovsky, 2004; Jackson et al., 2012; reviewed in Masland, 2012; reviewed in Popova, 2014).

Establishment of novel Tg(nr4a2a:eGFP) zebrafish line

A number of transgenic zebrafish lines exist that mark different populations of DA neurons driven by promoters of genes encoding for factors such as dopamine transporters, tyrosine hydroxylase, orthopedia homolog b (Gao et al., 2005; Meng et al., 2008; Wen et al., 2008; Bai and Burton, 2009; Fujimoto et al., 2011; Xi et al., 2011). These lines are extremely useful for labelling DA neurons, but if we are to understand how DA neurons are first generated or differentiated, it is

important to characterise the expression of genes that are involved in the earliest stages of DA neuron specification and differentiation. Here, we generated a novel transgenic line using the promoter of the *Nr4a2a* gene that is expressed very early in neuron development to be used in conjunction to the specific DA lines to visualise DA neurons from the very beginning. This allows us to perform time-lapse experiments, where cells that are identified to be dopaminergic by late differentiation markers at later time-points can then be tracked back in time and followed by expression of the eGFP in this transgenic line. Cells can be tracked in genetic studies, which affect early development and/or differentiation of neurons at stages at which they do not yet express the final DA machinery, including for example the dopamine transporter and tyrosine hydroxylase. Here, we utilised this line to study the expression of *Nr4a2a*. Alterations in *Nr4a2a* expression can be compared using gene knockout or overexpression studies within this population to clarify the causes of DA neuron loss. By following the transgenically labelled cells, we can assess cell fate, cell number, contributions of apoptosis, differentiation arrest and the features of DA neurons that rely on *Nr4a2a*. Understanding the dynamic expression of individual genes such as *Nr4a2a* may drive forward therapeutic treatment.

Within the retina, the fluorescent eGFP reporter expression matches the timeline of endogenous RNA expression and co-labels in individual retinal cells. As expected, our detailed time course analyses additionally shows eGFP labelled cells that do not show mRNA signal, revealing that the stable eGFP reporter can be visualised in cells that might only activate the endogenous *nr4a2a* promoter transiently. Consistently, we observe a gradual increase of cells labelled by our transgene in appropriate layers suggesting that the more stable eGFP perdures throughout retinogenesis. At 5 dpf, when visual circuits are functional (Easter and Nicola, 1996; 1997; Schmitt and Dowling, 1999), we still see little decrease in the number of eGFP cells, although we cannot rule out that a few cells may no longer express the transgene. Thus, all cells that express *Nr4a2a* throughout development can be followed. This includes the expression in the ganglion cell layer, which has also been observed in the *Tg(dat:EGFP)* zebrafish line (Xi et al., 2011) and the mouse

retina (Jiang and Xiang, 2009). While we find similar co-labelling of endogenous *nr4a2a* mRNA and the eGFP transgene as well as eGFP-only labelled cells in other CNS regions, the remainder of the discussion focuses only on the retina. A thorough time-course analysis of co-labelling for relevant CNS areas of interest should be conducted as required, to determine whether the transgene faithfully recapitulates *Nr4a2* expression in these regions.

Developmental lineage specific expression of Tg(nr4a2a:eGFP) in the retina

The expression of *nr4a2a* RNA and *Nr4a2a:eGFP* in the INL is detected after amacrine cell differentiation has begun (Jusuf and Harris, 2009). The time-lapse imaging confirms, that these cells have exited the cell cycle and completed migration to the amacrine layer. This is consistent with the lack of cell cycle labelling including 5-Bromo-2'-deoxyuridine DNA synthesis labelling, phospho-histone H3 M-phase marker, or proliferating cell nuclear antigen labelling of *Nr4a2* expressing cells in the rodent retina (Jiang and Xiang, 2009; Li et al., 2009; Li et al., 2012).

In contrast to previous zebrafish retinal *nr4a2a* expression data, a transgenic zebrafish line labelling differentiated DA neurons by driving reporter GFP under the dopamine transporter promoter also expressed in the GCL, though these might be displaced amacrine cells (see Figure 4B, 7A Xi et al., 2011). Another zebrafish line, in which a rat *th* promoter drives expression of a reporter GFP also shows widespread expression across most of the amacrine and ganglion cell layer, though how this can be reconciled with the much more limited TH antibody staining remains unclear (Gao et al., 2005). Consistent with our expression data, in the mouse retina, *nr4a2 in situ* signal was also observed more numerous in the GCL (Jiang and Xiang, 2009). We believe that transgenically labelled cells within the GCL faithfully recapitulate *nr4a2a* promoter activation, with our *in situ* expression indicating that *Nr4a2a* is expressed, though extremely transiently, in this layer.

Using *in vivo* time-lapse imaging we characterised a stereotypical lineage of *Nr4a2a* retinal neurons. *Nr4a2a:eGFP* expression in all retinal layers (*i.e.* inner nuclear layer and ganglion cell

layer) was exclusively within strongly labelled Atoh7 cells. Strong Atoh7 expression defines a specific progenitor subpopulation that gives rise to all ganglion cells as well as subpopulations of other neurons including amacrine cells (Poggi et al., 2005; Jusuf et al., 2011; Jusuf et al., 2012). Nr4a2a:eGFP expression specifically in the inner nuclear layer additionally only occurred in cells from the Ptf1a lineage. Thus Nr4a2a expression is limited to a subpopulation of amacrine cells including those we previously showed to arise from the Atoh7 lineage (Jusuf et al., 2011).

● Ganglion and amacrine cells start differentiation in the zebrafish retina at 28 hpf and 35 hpf, respectively (Kay et al., 2001; Poggi et al., 2005; Jusuf and Harris, 2009), significantly before any *nr4a2a* mRNA or Nr4a2a:eGFP expression can be detected. This indicates that Nr4a2a is unlikely to play an important role in cell cycle exit and functions later at the stages of subtype diversification or terminal differentiation of neurons.

Nr4a2a:eGFP expressing in DA and non-DA neuron subtypes

Due to its implication for DA neurons, the main focus in the literature has been on the expression and role of Nr4a2 for this particular cell fate. Indeed we find that tyrosine hydroxylase immunoreactive cells (marker of DA neurons across all CNS regions) are indeed co-labelled with the Nr4a2a:eGFP in our line. Within the retina, we did not distinguish between the two described subpopulations of tyrosine hydroxylase immunoreactive cells (Jang et al., 2011), though our results are consistent with Nr4a2a expression in both of these populations. Additionally, Nr4a2 seems to be expressed in a distinct subpopulation of amacrine interneurons in the retina (rather than randomly in a similar percentage of each subtype). This was strikingly exemplified by the lack of Nr4a2 for most of the subtype markers tested. Beside TH, Nr4a2 cells in the retina seem to be exclusively expressed in other GABAergic cells, though not all GABAergic cells express Nr4a2. This pattern closely resembles the expression of the Barhl2 transcription factor, which is also confined to this lineage and can specifically drive GABAergic phenotype (Jusuf et al., 2012). It will be interesting to investigate the relationship in the expression of Nr4a2 and Barhl2 and assess whether they work

independently or together to specify these amacrine cell fates. In other CNS regions, we also observed Nr4a2 transgene expression primarily within GABAergic cells, though there were many more GABAergic cells that did not express Nr4a2. Thus, there may be region specific subtype distributions of Nr4a2.

We unexpectedly identified three relatively separate Nr4a2a expression profiles consisting of weak, medium or strong GFP expression rather than a normal distribution of a continuum of transgene expression, which could simply reflect expression variation. This was observed in every animal examined, and within a given animal, the three different transgene intensities identified subjectively correlated 100% with objective ImageJ measurements. The only small overlap observed in the graph in Figure 10 is due to differences between retinas, in which measurements of the brightest “weak” cell in one retina may be larger than the measurement of the dimmest “medium” cell in another retina. Differences between retinas could also arise if some animals are homozygous for GFP, i.e. leading to a relative brighter expression than in heterozygous animals. Given the limited subtype markers available to label amacrine populations in zebrafish, we were not able to identify a strong correlation between GFP intensity and specific subtype. It will be interesting to correlate intensity with different aspects of subtypes (not only immunohistochemical markers, but perhaps morphology) and to test whether the level of Nr4a2 expression (with brighter cells either activating transcription at a higher level or for a longer developmental period) has different functions during neurogenesis and perhaps distinct effects on different subtypes.

There are also many neurons across the central nervous system that express Nr4a2 in mammals and Nr4a2a/b in the zebrafish, but do not generate DA neurons (Filippi et al., 2007). This is at least partially explained by the co-ordinated role Nr4a2 is believed to play with other factors such as Pitx3 and Fox2a (reviewed in Smidt and Burbach, 2009; Lee et al., 2010). However, within the retina, the DAC do not express the same co-factors, such as Pitx3. Thus, the question remains, what the exact role of Nr4a2 is during development, subtype fate specification, maintenance or cell survival, noting that its role may differ depending on the CNS region. Given that Nr4a2a is

expressed early enough during development to influence all of these aspects of development and maintenance within the retinal neurons as we describe here, its expression in non-DA subpopulations of cells shows that Nr4a2a at least within this tissue is not sufficient to drive the full DA subtype fate. This may reflect additional factors being necessary or point towards the importance of maintaining its expression to prevent alternate fates. This is a particularly important result to consider given the regenerative efforts that are already using overexpression of Nr4a2 by itself to specifically drive DA neuron generation (Lei et al., 2011; Hong et al., 2014). Important co-factors, which may be CNS region-specific, need to be identified to drive these efforts further.

Conclusions

Nr4a2a is expressed in both DA and non-DA (primarily GABAergic) neurons in the vertebrate retina. By using a novel Nr4a2a:eGFP transgenic zebrafish line cells that express Nr4a2a at some stage of their development, even if only very transiently, were found to originate from a specific progenitor lineage. Following the expression analysis presented here, we can now gain a better understanding of the role of Nr4a2 using the advantages of the zebrafish model to visualise and follow these developing cells *in vivo*. It will be particularly useful to characterise its specific role in DA neurons, as the Nr4a2 orphan receptor has already been implicated in DA neurogenesis. The expression of Nr4a2 in non-DA neurons is consistent with other labelling studies across vertebrates and marking all Nr4a2 expressing neurons in this line will allow us to better understand, which differences at which developmental stage contribute to the generation of a DA or non-DA neuron. This may reveal complex patterns of co-expression with other factors or some timing / expression level differences. We can directly assess the role that Nr4a2a and other genes play during fate specification and later differentiation stages. Focusing on other non-DA subtypes expressing Nr4a2a in this transgenic line will be useful for identifying related cell fates that might be reprogrammed into DA neurons.

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Conflict of interest statement

The authors have no conflict of interest.

Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. This project was carried out in the laboratories of WAH & PDC. PJ, JH generated the transgenic line, PJ performed experiments Figure 1 – 7, AW carried out antibody specificity experiments, LG & AW designed and performed experiments Figures 8 – 10. All authors contributed to manuscript preparation.

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Figure Legends

Figure 1. Comparison of *Tg(nr4a2a:eGFP)* expression and *nr4a2a* endogenous *in situ* RNA signal in wholemount zebrafish embryos. Robust *nr4a2a in situ* signal is observed in various central nervous system areas at 48 hpf (A, B), 72 hpf (E, F, F' lateral) and 96 hpf (I, J). At equivalent time, strong *Nr4a2a:eGFP* expression is present in many of the same areas (C, D, G, H, I, J). Additional *eGFP* expression is evident in some areas at 48 hpf (C, D compared to A, B) and more obviously so at 72 hpf (G, H compared to E, F) and 96 hpf (K, L compared to I, J), when *nr4a2a in situ* signal is starting to decrease in many areas. Arrowheads at 96 hpf show large areas of the hindbrain and spinal cord that no longer show RNA expression, but are still labelled with the *Nr4a2a:eGFP* transgene. FB: Forebrain; Ret: Retina; OT: Optic tectum; MB: Midbrain; HB: Hindbrain; SC: Spinal cord. Scale bar in L (A – L) is 100 μ m.

Figure 2. Temporal-spatial expression of *Tg(nr4a2a:eGFP)* transgenic fish line in wholemount zebrafish embryos. Left column shows lateral view, right column shows dorsal view for all time points. Faint *Nr4a2a:GFP* expression is first observed at 24 hpf and increases in different regions of the central nervous system as developmental time progresses (A – G). The reporter transgene remains during this time, such that any expressing CNS region maintains strong *eGFP* labelling. By 60 hpf strong *eGFP* expression can be observed in distinct retinal neurons (G higher power inset). FB: Forebrain; Ret: Retina; MB: Midbrain; HB: Hindbrain; SC: Spinal cord. Scale bar G (A – G) is 200 μ m, scale bar G inset is 100 μ m.

Figure 3. Co-labelling of *Nr4a2a:GFP* transgene and endogenous *nr4a2a* mRNA in the retina. Section *in situ* hybridisation of *nr4a2a mRNA* visualised by Fast Red combined with *GFP* immunohistochemistry in our *Tg(nr4a2a:eGFP)* line at 5 dpf. A – A'' Endogenous *nr4a2a* mRNA expression is maintained in a subpopulation of amacrine cells in the INL (red in A, A'').

Nr4a2a:GFP expression at this age is restricted to a subpopulation of amacrine cells in the INL and additionally in some cells in the GCL (green in A', A''). The merged channel (A'') reveals co-labelling of GFP in Fast Red labelled cells as well as additional GFP only expressing cells, that presumably no longer express the endogenous mRNA. **a – a''** High power inset of box shown in A – A''. Co-labelling at single cell resolution can be easier seen at high power, where double labelled cells are indicated by asterisks. **B – B''** High power view of a different section showing the same result, with cells in the INL expressing the endogenous *nr4a2a mRNA* (red) co-labelling with the Nr4a2a:GFP transgene (green) as indicated by asterisks. INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar A (A – A'') is 20 μm , scale bar a (a – a'', B – B'') is 10 μm .

Figure 4. Temporal expression of *nr4a2a mRNA in situ* signal and *Tg(Nr4a2a:eGFP)* transgene in retinal sections of developing zebrafish embryos. **A – G** Endogenous *nr4a2a mRNA* is expressed primarily in developing cells in the inner half of the inner nuclear layer, where mature amacrine interneurons reside. When neural differentiation begins at 28 hpf, there are no *nr4a2a* expressing cells. Expression can be seen robustly starting at 40 hpf (B). As development progresses, the number of cells expressing *nr4a2a mRNA* reduces. **H – N** Nr4a2a:eGFP is also first observed in very few cells at 40 hpf. As development progresses, Nr4a2a:eGFP is also expressed most strongly in the amacrine layer mirroring the mRNA expression. The stable eGFP continues to be expressed longer than the transient mRNA, such that at 120 hpf (N compared to G) there are still more GFP+ cells compared to those expressing endogenous *nr4a2a mRNA*. Additionally Nr4a2a:eGFP expression is also observed in numerous cells of the future ganglion cell layer, where mRNA signal is rarely observed, suggesting either labelling of additional neurons in the transgenic line or labelling of cells express the endogenous mRNA only very transiently. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Scale bar N (A – N) is 50 μm .

Figure 5. Temporal expression of Nr4a2a:eGFP during *live* development within the amacrine lineage. **A – H** Micrographs (2 hourly) from a time-lapse movie of double transgenic Tg(*ptf1a*:RFP / *nr4a2a*:eGFP) zebrafish embryo. The brightness of the entire figure is adjusted simultaneously, such that individual panels are directly comparable. Ptf1a:RFP expression in developing inhibitory neurons can be observed at 35 hpf. At this time, Nr4a2a:eGFP transgene starts being expressed in Ptf1a:RFP unlabelled ganglion cells (A, B). By 40 hpf (C, D), some of the inner nuclear layer cells start expressing Nr4a2a:eGFP labelled weakly. Expression increases in this layer as development progresses. Ptf1a:RFP labelled horizontal cells that start migrating back apically around 45 hpf (F) do not express Nr4a2a:eGFP. **I** Micrograph of a 45 hpf snapshot of the movie. Magnified inset reveals that Nr4a2a:eGFP expressing cells in the inner nuclear layer co-label with Ptf1a:RFP (white dots). HC: horizontal cells; AC: amacrine cells; GC: ganglion cells. Scale bar H (A – H) is 50 μ m, scale bar I is 50 μ m, scale bar I''' (I' – I''') is 10 μ m.

Figure 6. Expression of Nr4a2a:eGFP and *nr4a2a* mRNA in some neurons in the ganglion cell layer of the retina. **A, B** Expression of transgenic Nr4a2a:eGFP consistently labels cells in the amacrine layer (ACL) and additionally cells in the ganglion cell layer (GCL) as seen in 5 dpf retinal sections. **C – H** Even though rarely observed in retinal sections at 5 dpf, *nr4a2a in situ* labelled cells can be observed in GCL at earlier 48 hpf or 72 hpf in wholemount (C – E) and much more rarely in retinal sections (F – H), suggesting that eGFP in this layer may represent perdurance of the stable transgene in cells that express the endogenous transcript only very transiently. **F' – H'** Higher magnification insets of boxes shown in F – H. Arrowheads indicate *nr4a2a* mRNA labelling detected by *in situ* hybridisation in individual cells in the ganglion cell layer. Scale bar B (A, B) is 50 μ m, scale bar E (C – E) is 50 μ m, scale bar H (F – H) is 50 μ m, scale bar H' (F' – H') is 10 μ m.

Figure 7. *Live* expression of Nr4a2a:eGFP exclusively within the Atonal 7 lineage in all retinal layers. **A** Micrograph of a 45 hpf snapshot of a movie of double transgenic Tg(*atoh7*:RFP /

nr4a2a:eGFP) zebrafish embryo. Nr4a2a:eGFP expression in both the ganglion cell layer (GCL) and amacrine cell layer (ACL) in the inner nuclear layer co-label with the membrane Atoh7:RFP transgene. Magnified inset shows co-labelled cells in the GCL and (white dots). Scale bar A is 50 μm , scale bar A''' is 10 μm .

Figure 8. Co-labelling of *Tg(nr4a2a:eGFP)* and tyrosine hydroxylase immunoreactivity in retinal sections of 5 dpf zebrafish embryos. **A – C** DAC in the retina labelled with tyrosine hydroxylase immunohistochemistry were also marked by a small population of the Nr4a2a:GFP labelled amacrine cells (asterisks indicate double labelled cells in the insets showing boxed area at higher magnification). Scale bar C (A – C) is 20 μm , scale bar C' (A' – C''') is 10 μm .

Figure 9. Immunohistochemical co-labelling of *Tg(nr4a2a:eGFP)* with non-dopaminergic amacrine subpopulation markers in retinal sections of 5 dpf zebrafish embryos.

A – H Micrographs showing retinal overview (left panel) and higher power view of individual as well as combined red and green channels of boxed regions for each of the amacrine subtype markers used. **A** Some of the GABAergic amacrine interneuron subpopulations were co-labelled with cells expressing varying degrees of Nr4a2a:eGFP (white dots), though GABAergic amacrine cells that did not express any Nr4a2a:eGFP were also observed (white asterisks). **B – H** For the vast majority of amacrine subtype or subpopulation markers, there was very little co-labelling with Nr4a2a:eGFP (white dots), with the majority of immunohistochemically labelled cells not expressing any detectable Nr4a2a:eGFP (white asterisks). INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar in left panel H (A – H) is 50 μm , scale bar inset H (A – H) is 10 μm .

Figure 10. Co-labelling of amacrine subtype markers with three distinct intensities of *Tg(nr4a2:eGFP)* transgene labelling. **A** Micrograph of retinal sections from zebrafish at 5 days post

fertilisation (dpf) reveal different intensity of transgene staining (1 – weak, 2 medium, 3 strong GFP in higher power inset A' of boxed region in A). **B** Graph showing subjective categorization (1 – 3) and corresponding intensity measurement. The transgene intensity differences quantitatively (0 – 255) fall into three distinct populations (three peaks with dips in-between), which match subjective classification well. The small overlap that can be observed in the graph is due to variations between samples. Categorization within individual images never showed any such overlap. **C** Using the three intensities, the co-localisation of Nr4a2a with various amacrine subtype markers was quantified. As expected Nr4a2a completely co-labelled with tyrosine hydroxylase immunoreactive dopaminergic neurons, which primarily expressed a medium level of Nr4a2a:eGFP transgene. Nr4a2a:eGFP was expressed to varying degrees in the multiple GABAergic amacrine subpopulations, but showed only very minimal co-expression with any other established amacrine subtype marker including the four calcium binding proteins parvalbumin (PV), calretinin, secretagogin (SCGN) and calbindin, as well as choline acetyltransferase (ChAT), neuropeptide Y (NY) and serotonergic (5HT) labelled amacrine cells. n = number of cells analysed for each immunohistochemical marker. INL: inner nuclear layer; GCL: ganglion cell layer. Scale bar A is 50 μm , scale bar inset A is 10 μm .

Table 1. Primary antibodies used

Antigen	Immunogen	Source, host species, catalog No., RRID	Dilution
Calbindin-D-28K	Purified bovine cerebellum calbindin D-28K protein	Merck-Millipore, rabbit IgG polyclonal, PC253L, AB_213554	1:500
Calretinin	Recombinant rat calretinin	Merck-Millipore, rabbit polyclonal, AB5054, AB_2068506	1:2000
ChAT	Human placental enzyme anti-choline acetyltransferase	Merck-Millipore, goat polyclonal, AB144P, AB_2079751	1:500
GABA	Gamma-aminobutyric acid conjugated to BSA	Sigma, rabbit polyclonal, A2052, AB_477652	1:2000
GFP	Green fluorescent protein isolated from jellyfish <i>Aequorea victoria</i>	Invitrogen Lifetech, chicken IgY polyclonal, A10262, AB_2534023	1:400
NY	Neuropeptide Y coupled to bovine thyroglobulin with glutaraldehyde	Immunostar, rabbit IgG polyclonal, 22940, AB_572253	1:500
PV	Parvalbumin purified from frog muscle	Merck-Millipore, mouse IgG1 monoclonal, MAB1572, clone PARV-19, AB_2174013	1:1000
Secretagogin	Full length (276AA) recombinant human secretagogin + 10AA N-term histidine-tag	Biovendor R&D, rabbit polyclonal, RD181120100, AB_2034060	1:4000
Secretagogin	Full length (276AA) recombinant human secretagogin + 10AA N-term histidine-tag	Biovendor R&D, sheep polyclonal, RD184120100, AB_2034062	1:4000
TH	Tyrosine hydroxylase purified from PC12 cells	Merck-Millipore, mouse IgG1 monoclonal, MAB318, clone LNC1, AB_2201528	1:1000

5HT	Serotonin creatinine sulphate complex conjugated with formaldehyde to BSA.	Sigma, rabbit polyconal, S5545, AB_477522	1:500
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Accepted Article

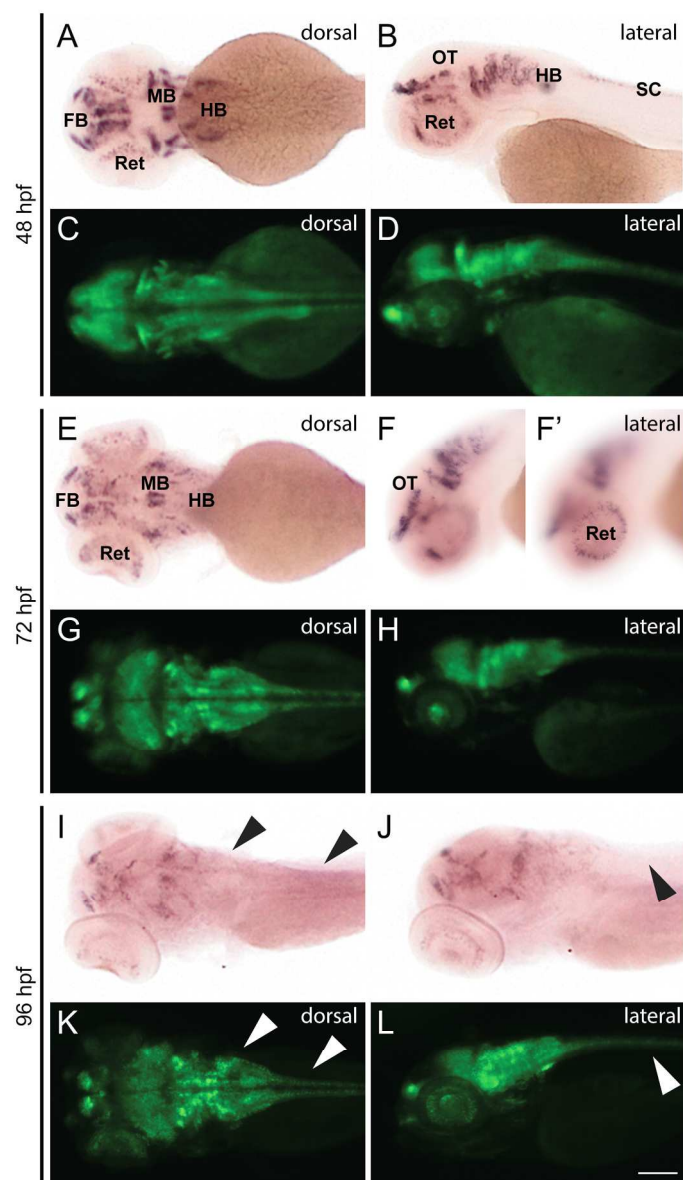


Figure 1. Comparison of Tg(nr4a2a:eGFP) expression and nr4a2a endogenous in situ RNA signal in wholemount zebrafish embryos. Robust nr4a2a in situ signal is observed in various central nervous system areas at 48 hpf (A, B), 72 hpf (E, F, F' lateral) and 96 hpf (I, J). At equivalent time, strong Nr4a2a:eGFP expression is present in many of the same areas (C, D, G, H, I, J). Additional eGFP expression is evident in some areas at 48 hpf (C, D compared to A, B) and more obviously so at 72 hpf (G, H compared to E, F) and 96 hpf (K, L compared to I, J), when nr4a2a in situ signal is starting to decrease in many areas. Arrowheads at 96 hpf show large areas of the hindbrain and spinal cord that no longer show RNA expression, but are still labelled with the Nr4a2a:eGFP transgene. FB: Forebrain; Ret: Retina; OT: Optic tectum; MB: Midbrain; HB: Hindbrain; SC: Spinal cord. Scale bar in L (A – L) is 100 μ m.

Figure 1

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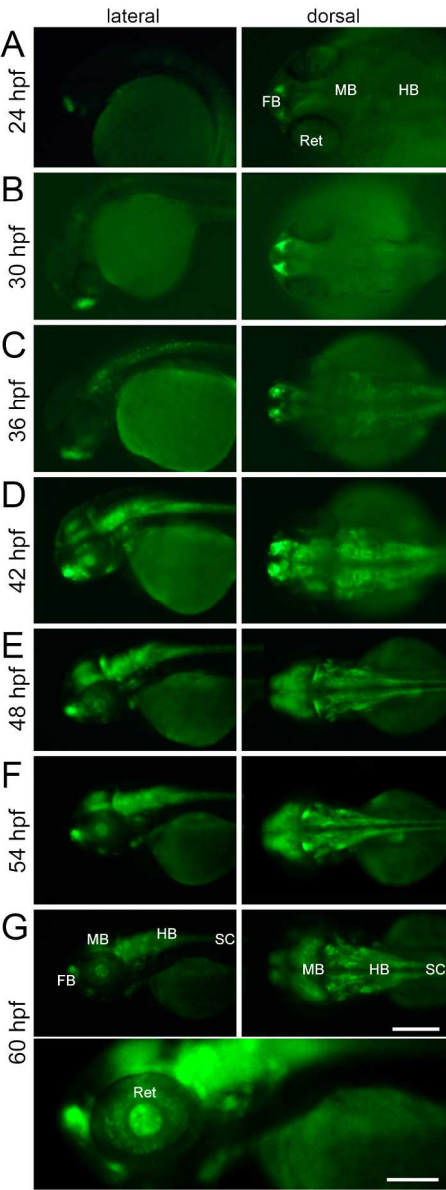


Figure 2. Temporal-spatial expression of Tg(nr4a2a:eGFP) transgenic fish line in wholemount zebrafish embryos. Left column shows lateral view, right column shows dorsal view for all time points. Faint Nr4a2a:GFP expression is first observed at 24 hpf and increases in different regions of the central nervous system as developmental time progresses (A – G). The reporter transgene remains during this time, such that any expressing CNS region maintains strong eGFP labelling. By 60 hpf strong eGFP expression can be observed in distinct retinal neurons (G higher power inset). FB: Forebrain; Ret: Retina; MB: Midbrain; HB: Hindbrain; SC: Spinal cord. Scale bar G (A – G) is 200 μ m, scale bar G inset is 100 μ m.

Figure 2

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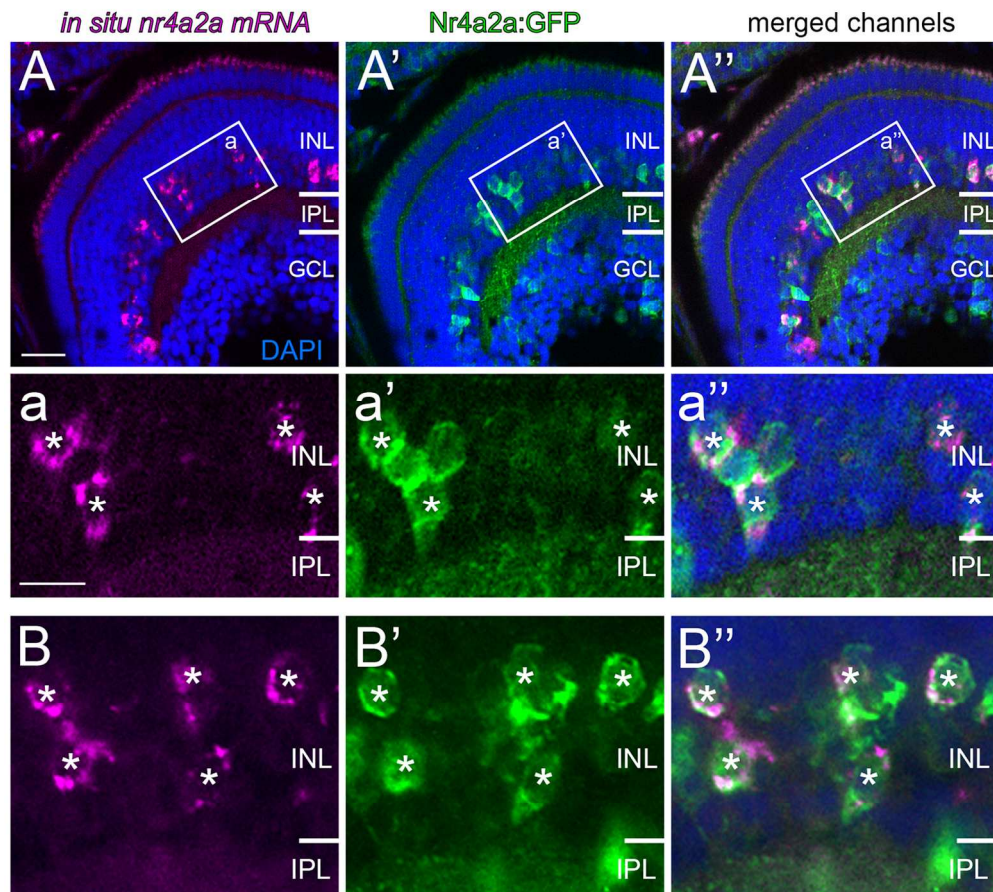


Figure 3. Co-labelling of Nr4a2a:GFP transgene and endogenous nr4a2a mRNA in the retina. Section in situ hybridisation of nr4a2a mRNA visualised by Fast Red combined with GFP immunohistochemistry in our Tg(nr4a2a:eGFP) line at 5 dpf. A – A'' Endogenous nr4a2a mRNA expression is maintained in a subpopulation of amacrine cells in the INL (red in A, A''). Nr4a2a:GFP expression at this age is restricted to a subpopulation of amacrine cells in the INL and additionally in some cells in the GCL (green in A', A''). The merged channel (A'') reveals co-labelling of GFP in Fast Red labelled cells as well as additional GFP only expressing cells, that presumably no longer express the endogenous mRNA. a – a'' High power inset of box shown in A – A''. Co-labelling at single cell resolution can be easier seen at high power, where double labelled cells are indicated by asterisks. B – B'' High power view of a different section showing the same result, with cells in the INL expressing the endogenous nr4a2a mRNA (red) co-labelling with the Nr4a2a:GFP transgene (green) as indicated by asterisks. INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar A (A – A'') is 20 μ m, scale bar a (a – a'', B – B'') is 10 μ m.

Figure 3

129x117mm (300 x 300 DPI)

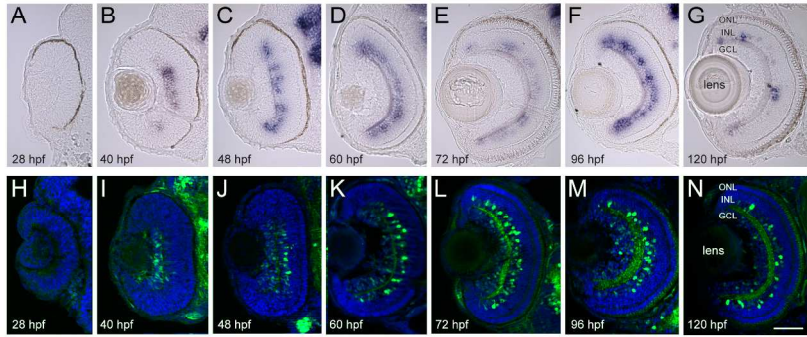


Figure 4. Temporal expression of *nr4a2a* mRNA in situ signal and Tg(*Nr4a2a*:eGFP) transgene in retinal sections of developing zebrafish embryos. A – G Endogenous *nr4a2a* mRNA is expressed primarily in developing cells in the inner half of the inner nuclear layer, where mature amacrine interneurons reside. When neural differentiation begins at 28 hpf, there are no *nr4a2a* expressing cells. Expression can be seen robustly starting at 40 hpf (B). As development progresses, the number of cells expressing *nr4a2a* mRNA reduces. H – N *Nr4a2a*:eGFP is also first observed in very few cells at 40 hpf. As development progresses, *Nr4a2a*:eGFP is also expressed most strongly in the amacrine layer mirroring the mRNA expression. The stable eGFP continues to be expressed longer than the transient mRNA, such that at 120 hpf (N compared to G) there are still more GFP+ cells compared to those expressing endogenous *nr4a2a* mRNA. Additionally *Nr4a2a*:eGFP expression is also observed in numerous cells of the future ganglion cell layer, where mRNA signal is rarely observed, suggesting either labelling of additional neurons in the transgenic line or labelling of cells express the endogenous mRNA only very transiently. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Scale bar N (A – N) is 50 μ m.

Figure 4

237x118mm (300 x 300 DPI)

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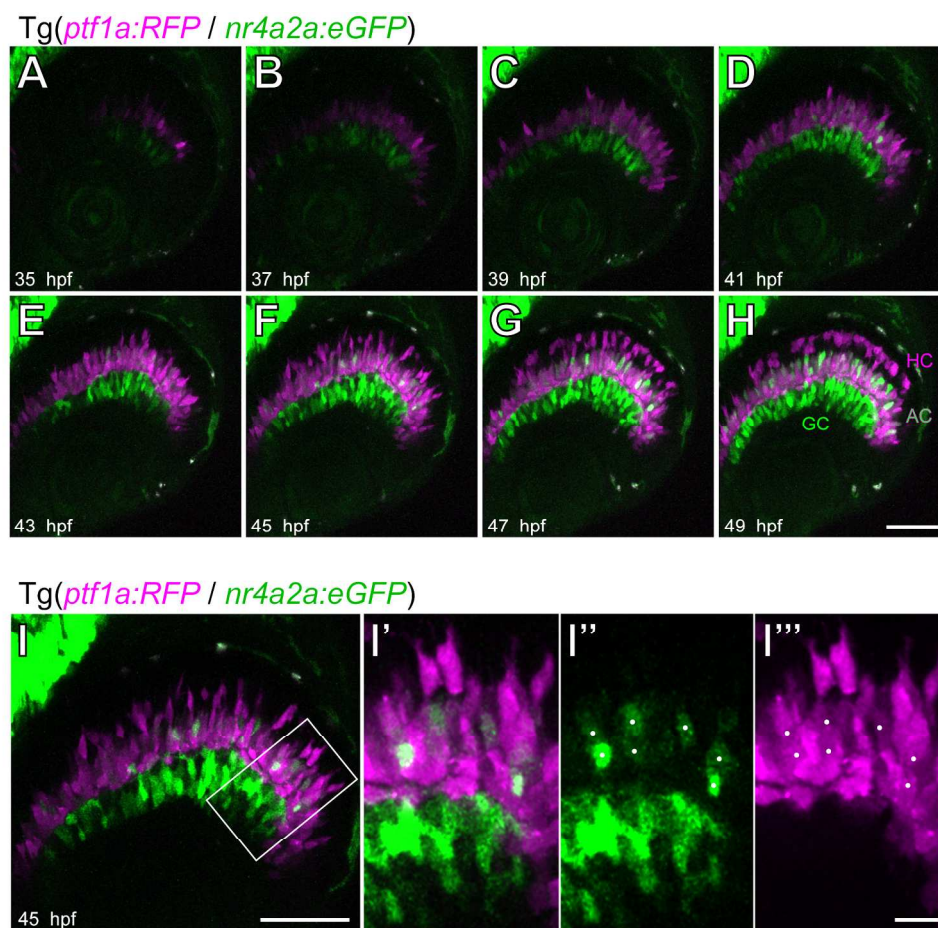


Figure 5. Temporal expression of Nr4a2a:eGFP during live development within the amacrine lineage. A – H Micrographs (2 hourly) from a time-lapse movie of double transgenic Tg(*ptf1a:RFP* / *nr4a2a:eGFP*) zebrafish embryo. The brightness of the entire figure is adjusted simultaneously, such that individual panels are directly comparable. Ptf1a:RFP expression in developing inhibitory neurons can be observed at 35 hpf. At this time, Nr4a2a:eGFP transgene starts being expressed in Ptf1a:RFP unlabelled ganglion cells (A, B). By 40 hpf (C, D), some of the inner nuclear layer cells start expressing Nr4a2a:eGFP labelled weakly. Expression increases in this layer as development progresses. Ptf1a:RFP labelled horizontal cells that start migrating back apically around 45 hpf (F) do not express Nr4a2a:eGFP. I Micrograph of a 45 hpf snapshot of the movie. Magnified inset reveals that Nr4a2a:eGFP expressing cells in the inner nuclear layer co-label with Ptf1a:RFP (white dots). HC: horizontal cells; AC: amacrine cells; GC: ganglion cells. Scale bar H (A – H) is 50 μ m, scale bar I is 50 μ m, scale bar I''' (I' – I'') is 10 μ m.

Figure 5
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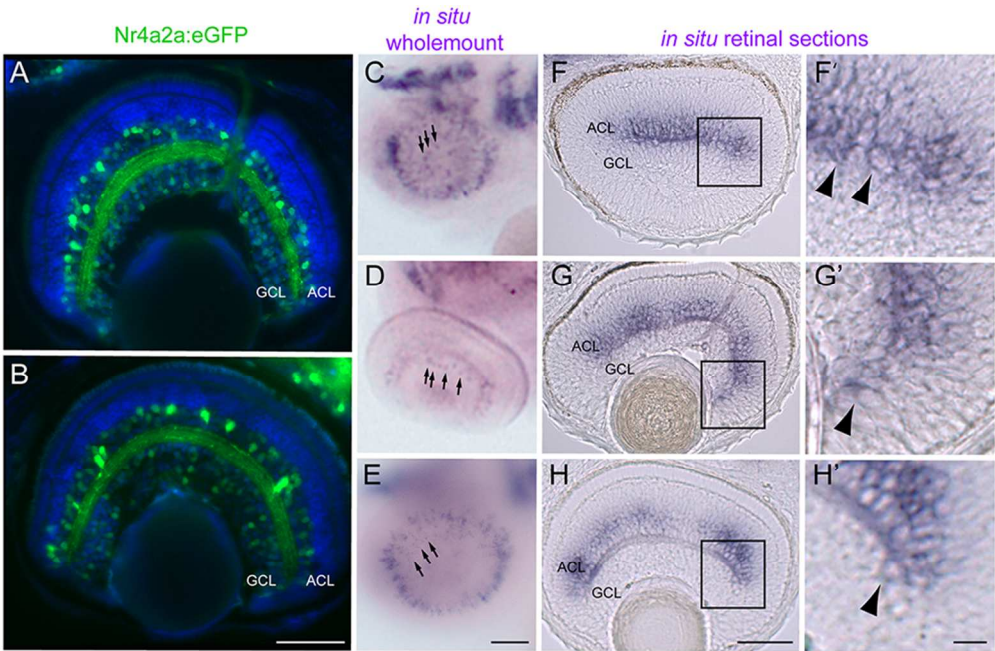


Figure 6. Expression of Nr4a2a:eGFP and nr4a2a mRNA in some neurons in the ganglion cell layer of the retina. A, B Expression of transgenic Nr4a2a:eGFP consistently labels cells in the amacrine layer (ACL) and additionally cells in the ganglion cell layer (GCL) as seen in 5 dpf retinal sections. C – H Even though rarely observed in retinal sections at 5 dpf, nr4a2a in situ labelled cells can be observed in GCL at earlier 48 hpf or 72 hpf in wholemount (C – E) and much more rarely in retinal sections (F – H), suggesting that eGFP in this layer may represent perdurance of the stable transgene in cells that express the endogenous transcript only very transiently. F' – H' Higher magnification insets of boxes shown in F – H. Arrowheads indicate nr4a2a mRNA labelling detected by in situ hybridisation in individual cells in the ganglion cell layer. Scale bar B (A, B) is 50 μ m, scale bar E (C – E) is 50 μ m, scale bar H (F – H) is 50 μ m, scale bar H' (F' – H') is 10 μ m.

Figure 6

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Acce

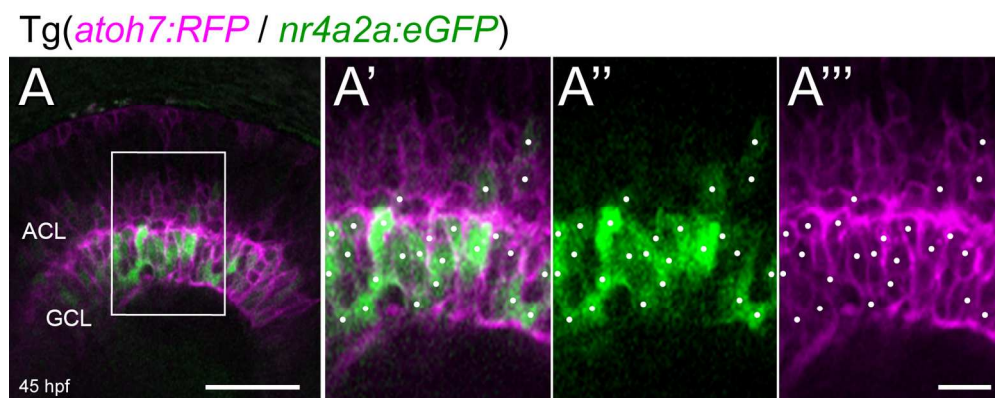


Figure 7. Live expression of Nr4a2a:eGFP exclusively within the Atonal 7 lineage in all retinal layers. A Micrograph of a 45 hpf snapshot of a movie of double transgenic Tg(*atoh7:RFP* / *nr4a2a:eGFP*) zebrafish embryo. Nr4a2a:eGFP expression in both the ganglion cell layer (GCL) and amacrine cell layer (ACL) in the inner nuclear layer co-label with the membrane Atonal 7:RFP transgene. Magnified inset shows co-labelled cells in the GCL and (white dots). Scale bar A is 50 μ m, scale bar A''' is 10 μ m.

Figure 7

155x63mm (300 x 300 DPI)

Accepted

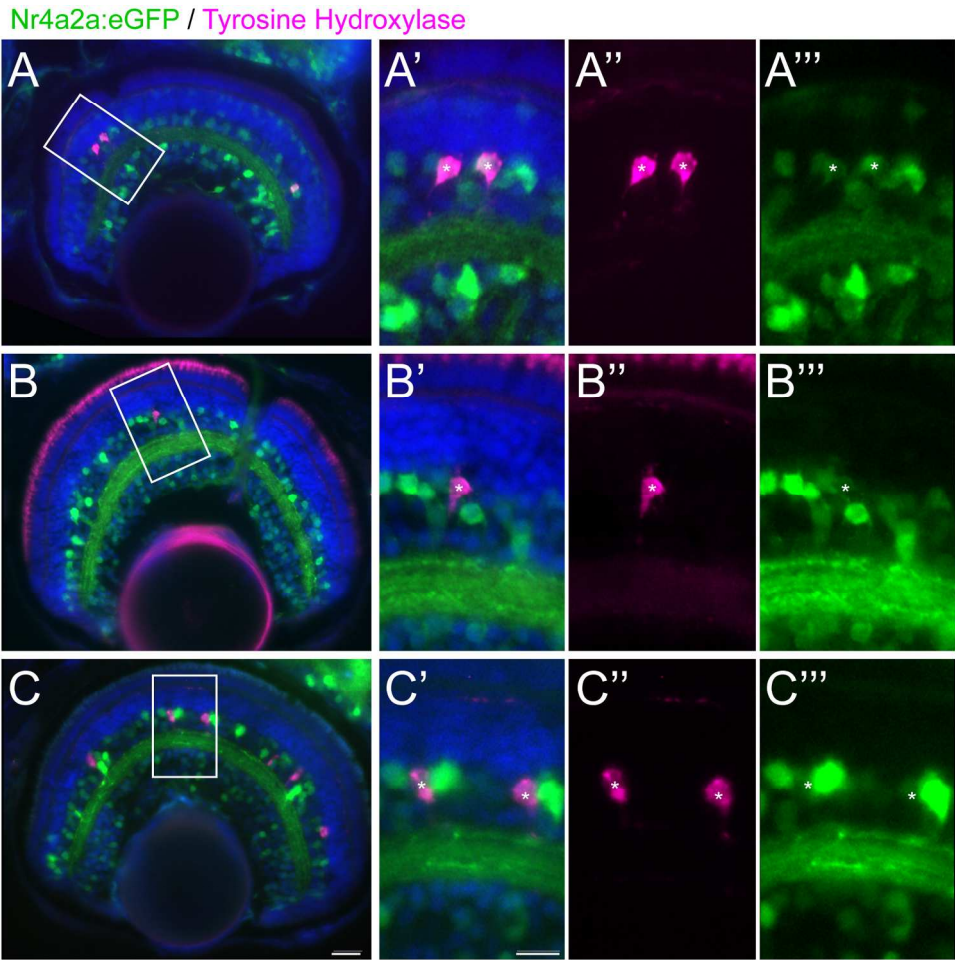


Figure 8. Co-labelling of Tg(nr4a2a:eGFP) and tyrosine hydroxylase immunoreactivity in retinal sections of 5 dpf zebrafish embryos. A – C DAC in the retina labelled with tyrosine hydroxylase immunohistochemistry were also marked by a small population of the Nr4a2a:GFP labelled amacrine cells (asterisks indicate double labelled cells in the insets showing boxed area at higher magnification). Scale bar C (A – C) is 20 μ m, scale bar C' (A' – C''') is 10 μ m.

Figure 8
179x175mm (300 x 300 DPI)

A

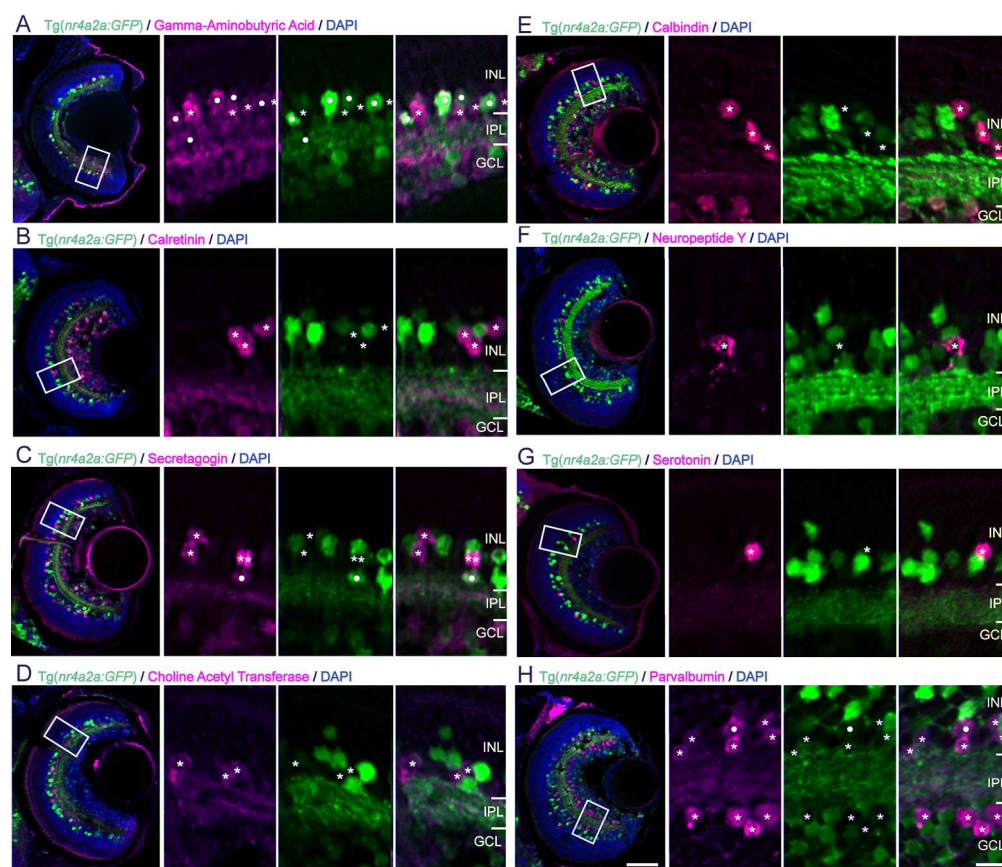
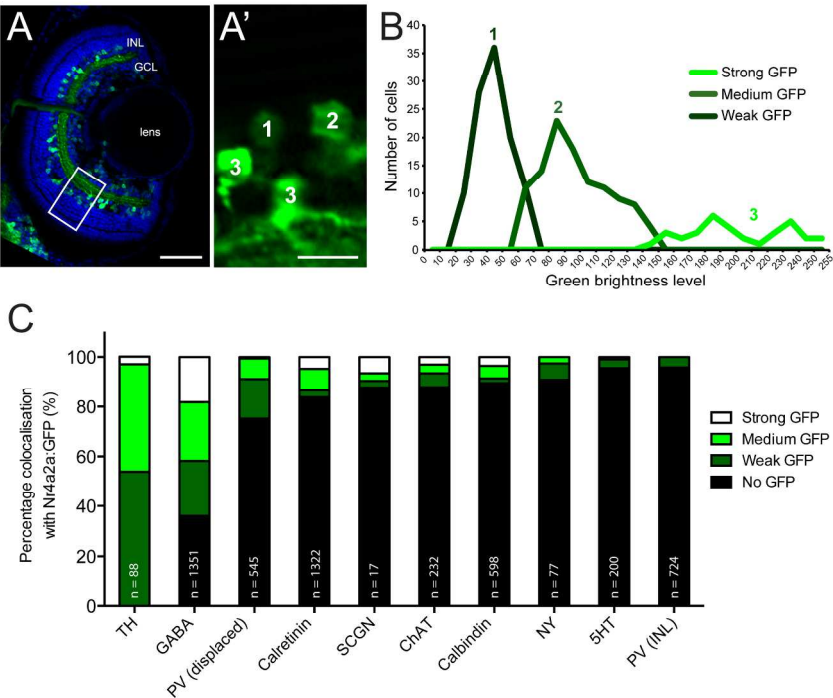


Figure 9. Immunohistochemical co-labelling of Tg(nr4a2a:eGFP) with non-dopaminergic amacrine subpopulation markers in retinal sections of 5 dpf zebrafish embryos. A – H Micrographs showing retinal overview (left panel) and higher power view of individual as well as combined red and green channels of boxed regions for each of the amacrine subtype markers used. A Some of the GABAergic amacrine interneuron subpopulations were co-labelled with cells expressing varying degrees of Nr4a2a:eGFP (white dots), though GABAergic amacrine cells that did not express any Nr4a2a:eGFP were also observed (white asterisks). B - H For the vast majority of amacrine subtype or subpopulation markers, there was very little co-labelling with Nr4a2a:eGFP (white dots), with the majority of immunohistochemically labelled cells not expressing any detectable Nr4a2a:eGFP (white asterisks). INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar in left panel H (A – H) is 50 μ m, scale bar inset H (A – H) is 10 μ m.

Figure 9
182x186mm (300 x 300 DPI)



Co-labelling of amacrine subtype markers with three distinct intensities of Tg(nr4a2:eGFP) transgene labelling. A Micrograph of retinal sections from zebrafish at 5 days post fertilisation (dpf) reveal different intensity of transgene staining (1 – weak, 2 medium, 3 strong GFP in higher power inset A' of boxed region in A). B Graph showing subjective categorization (1 – 3) and corresponding intensity measurement. The transgene intensity differences quantitatively (0 – 255) fall into three distinct populations (three peaks with dips in-between), which match subjective classification well. The small overlap that can be observed in the graph is due to variations between samples. Categorization within individual images never showed any such overlap. C Using the three intensities, the co-localisation of Nr4a2a with various amacrine subtype markers was quantified. As expected Nr4a2a completely co-labelled with tyrosine hydroxylase immunoreactive dopaminergic neurons, which primarily expressed a medium level of Nr4a2a:eGFP transgene. Nr4a2a:eGFP was expressed to varying degrees in the multiple GABAergic amacrine subpopulations, but showed only very minimal co-expression with any other established amacrine subtype marker including the four calcium binding proteins parvalbumin (PV), calretinin, secretagogin (SCGN) and calbindin, as well as choline acetyltransferase (ChAT), neuropeptide Y (NY) and serotonergic (5HT) labelled amacrine cells. n = number of cells analysed for each immunohistochemical marker. INL: inner nuclear layer; GCL: ganglion cell layer.

Scale bar A is 50 μ m, scale bar inset A is 10 μ m.

Figure 10
220x184mm (300 x 300 DPI)