Gata2, Nkx2-2 and Skor2 form a transcription factor network regulating development of a midbrain GABAergic neuron subtype with characteristics of REM-sleep regulatory neurons

Anna Kirjavainen, Parul Singh, Laura Lahti, Patricia Seja, Zoltan Lelkes, Aki Makkonen, Sami Kilpinen, Yuichi Ono, Marjo Salminen, Teemu Aitta-Aho, Tarja Stenberg, Svetlana Molchanova, Kaia Achim and Juha M. Partanen
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Reviewer 1

Evidence, reproducibility and clarity

Summary:
The reticular formation of the midbrain contains numerous clusters of inhibitory (GABAergic) and excitatory (glutamatergic) neurons. These groups of neurons are functionally diverse, but their developmental history and molecular profile are poorly studied. Previously, Juha Partanen's group has demonstrated with a series of elegant mouse genetic studies that the transcription factor Gata2 is required for the development of all embryonic midbrain-derived GABAergic neurons. The study by Kirjavainen et al. now shows that Gata2 regulates a number of transcription factors expressed in specific GABAergic subpopulations in the mouse midbrain. This includes, among others, the homeodomain transcription factor Nkx2-2 and the SKI family transcriptional repressor Skor2. Nkx2-2 and Skor2 are co-expressed in a small group of GABAergic precursor cells and both Gata2 and Nkx2-2 appear to be required for Skor2 expression. In the adult mouse and rat midbrain, the Skor2- positive GABAergic neurons are found at the boundary between the ventrolateral periaqueductal gray and the reticular formation of the midbrain. Since this very area contains REM-off neurons that regulate REM sleep, the authors investigate whether the Skor2-positive group contains these REM- off neurons. Indeed, c-FOS expression as an indicator of neuronal activity appears to be increased in Skor2-positive cells upon inhibition of REM sleep. Moreover, some Skor2-positive cells send projections to a pontine region associated with sleep control and they respond to orexins. These properties have also been found in REM-off neurons in the midbrain. In addition, the authors characterize the electrophysiological and morphological properties of Skor2-positive neurons and show that based on these characteristics, Skor2-positive neurons can be divided into several subgroups.

Major comments:
The majority of conclusions are well supported by the provided results. The following points would benefit from additional experiments, or the statements should be toned-down to reflect the experimental evidence:
1) Nkx2-2 knock-out: The authors conclude that Nkx2-2 is not required for cell survival of the neuronal population that would normally express Skor2 Cell death has only been tested at one developmental stage. Thus, it cannot be excluded that cell death occurs at later stages in the Nkx2-2 derived cells. Apoptosis could be assessed at additional developmental stages, or the conclusion could be toned down. The ideal experiment to exclude an effect on cell survival would

be to cross the Skor2-GFP allele into the Nkx2-2 background. However, this would be a time and resource consuming experiment and is thus beyond the scope of the current study.

2) Skor2 GFP/GFP mice, Figure 3M,M'. It appears that the knock-out contains more GFP positive cells, at least in the sections shown here. Have the authors investigated this? Quantification of cell numbers could be useful.

3) Expression of Skor2 in cerebellar Purkinje cells: The image shown in the Figure is overexposed. The authors should provide a better image (appropriate exposure, higher magnification of the cerebellum) to support this statement.

4) The authors claim that they „ followed the development of the neuronal precursors expressing Skor2 and Nkx2-2, asking whether they contribute to unique midbrain GABAergic nuclei in the postnatal brain"
Since the authors do not attempt fate-mapping of the Skor2 precursors this statement does not reflect the actual experiment. The authors analyze the Skor2-expressing population during embryogenesis and in the adult brain and assume that the Skor2-expressing precursors will develop into Skor2-expressing neurons. Moreover, it remains unclear whether the Skor2-expressing cells in the adult maintain Nkx2-2 expression. The statement should be re-phrased accordingly.

5) Abstract: "In the adult mouse as well as rat midbrain, the Nkx2-2 and Skor2 expressing GABAergic neurons locate at the boundary of the ventrolateral periaqueductal gray" and similar statements in the introduction (p.3) and results p.7/p.9
The authors do not show that the Skor2-positive cells express Nkx2-2 in the adult brain. The conclusion should be re-phrased accordingly.

6) Analysis of the co-expression of c-Fos and Skor2 revealed a significant increase in the proportion of c-Fos expressing dMRF/vlPAG Skor2+ neurons in the REM sleep deprived rats compared to the control groups or recovery group
The c-Fos expression is not convincing. c-Fos should fill out the nucleus, here it seems to be expressed in little speckles in the nucleus. The authors should provide better images and indicate with arrows which cells in these images they would consider positive.

7) Statement: Many of the CtB labelled cells showed co-expression of Skor2, suggesting that the Skor2+ dMRF/vlPAG neurons frequently project to the dorsolateral pons.
The results should be quantified (Skor2+/CTB+ and Skor2-/CTB+ cells) and the authors should indicate in the figure legend how many animals have been analyzed.

Presentation of data and methods, replication of experiments and statistical analysis are appropriate.

Minor comments:

Figure 4: The TH and NF staining are not mentioned in the text. This should be added.

The methods describe that retrobreads were used for retrograde tracing, but this is not mentioned in the results or Figures. This should be removed.

p.10 The authors state that „The number of responding cells correlated with the proportion of the Orexin receptor expressing Skor2+ cells." It is not clear what is meant here - comparing numbers and proportion?

The authors should highlight that Skor2 GFP/GFP mice die shortly after birth. Otherwise, the reader is left wondering why the authors did not explore the consequences of Skor2 inactivation on morphological and electrophysiological properties of the neurons or on REM sleep regulation.

Significance

The study by Kirjavainen et al. provides new insights into the developmental mechanisms that give rise to different groups of neurons in the ventral midbrain and a detailed characterization of a

small group of GABAergic neurons expressing the transcription factor Skor2. Because little is known about the development of different GABAergic neurons in the midbrain, this is a valuable contribution to the field of neurodevelopmental biology. The authors also hypothesize that the Skor2-positive cells correspond to REM-off neurons. The present data support this hypothesis but do not prove it beyond doubt. For example, it remains an open question whether Skor2+ cells are identical to REM-off neurons, are a subset of REM-off neurons, or REM-off neurons are a subset of Skor2 neurons. If Skor2-expressing neurons are identical to REM-off neurons, Skor2 would be a valuable marker for further exploration of this neuron population and REM sleep regulation (e.g., by generating Skor2- Cre mice and using them for Skor2-specific tracing of neuronal projections and for functional manipulation). An analysis of the electrophysiological and morphological properties of Skor2-GFP neurons in a Skor2 conditional knockout model and an assessment of the functional consequences of Skor2 inactivation on the regulation of REM sleep would provide important insight into the functional significance of Skor2 in defining the functional properties of this neuronal population.
Thus, this study lays the foundation for more detailed functional studies of this subset of GABAergic neurons in the midbrain.

Readership: neurodevelopmental researchers, researchers studying REM sleep, researchers studying the anatomical and functional diversity of neurons in the midbrain.

Reviewer, field of expertise: neurodevelopment, mouse genetics

Reviewer 2

Evidence, reproducibility and clarity

In this manuscript, Kaia Achim and Juha Partanen use cDNA arrays, progenitor labelling, conditional and constitutive mutagenesis and histological analysis to describe the development of a GABAergic neuronal subtype in the mouse midbrain that contributes to the ventrolateral periaqueductal gray and dorsomedial midbrain reticular formation (vlPAG/dMRF). This GABAergic subpopulation is defined by expression of Gata2, Nkx2.2 and Skor2 and the hierarchical regulation of these key transcription factors is also established. Using a classic (although with known limitations - see later) REM sleep deprivation assay in rats and retrograde tract tracing, they find indirect evidence that suggests a potential role for this developmentally defined neuronal population in the control of NREM-REM sleep transitions. It is suggested that the Nkx2.2/Skor2 neurons coincide - at least in part - with the REM-off neurons that a well-known model of sleep regulation (see Saper, Fort, Luppi and others) describes as engaging in reciprocal and antagonistic interactions with REM-on neurons in the pontine region. Cell membrane electrical properties for the neurons are also described, revealing an apparent degree of functional heterogeneity.

The data in this manuscript are generally of high quality, clearly presented and, importantly, novel. The molecular and developmental part of the paper does not rise any major concerns. Similarly, I find the classification of the electrical and morphological properties of the cells on acute brain sections sufficiently detailed for a first description and conductive to further in vivo studies - however, I am not an expert in this area.

The analysis of cFos positive neurons in the REM sleep deprivation study is possibly the weakest part of the manuscript. The authors hint at some inconsistent staining for this marker and the immunofluorescence images in Fig. 4 M to R' rise some concerns as to the specificity of the labelling. A suggestion would be to provide data for cFos staining in other areas of the brain to frame the level of activation detected in the Skor2 population against (presumably) higher levels of activation observed elsewhere.

Although beyond the scope of this paper, if the authors would have the opportunity to repeat the REM sleep deprivation study, they could consider combining it with the retrograde tracing from the dorsal pontine region to measure the ratio of cFos/Ctb/Skor2 triple positive cells.

The method of the "inverted flower pot" that relies on the REM associated muscle atonia to achieve REM sleep deprivation, also causes a significative increase in stress and is unlikely to selectively disrupt REM sleep, given the very short duration of REM episodes in rodents. New methods have been developed which relay on real time, close circuit EEG/EMG detection and optogenetic interference with REM sleep to achieve more accurate and specific REM deprivation (see for instance work from the Adamantidis lab). While clearly such and advanced approach goes beyond the scope of the manuscript, it may be possible for the authors to assess whether other behavioural paradigms that also lead to increased stress and anxiety show a similar increase in activation of the target Skort2 population.

Below are additional minor comments that, in my view, could help make the manuscript more accessible to a broader audience. In order of appearance in the manuscript.

Figure 1. It would be useful to see somewhere in the figure the schematic of the division of the midbrain neuroepithelium in "m" progenitor domains. This could be overlaid for instance on the image in C.

Figure 1B. it would be helpful to place an arrowhead also in L', similar to L.

Table S1. Please explain the colour code used.

Table S2. Check the headings, as currently not readable.

Related to Figure 1 and Supplementary Figure 1. Please clarify the criteria for selection of candidate genes used in qRT-PCR and ISH validation.

Related to Figure 1 and Supplementary Figure 1. Please comment on the results for Gata2. How is the discrepancy between fold change in RT-PCR and ISH/array explained?

Related to Figure 1 and Supplementary Table 1. In view of the known role of Gata2 as lineage selector gene, I would have expected to see glutamatergic lineage genes upregulated in the cKO sample. Why are these not included in the table? The authors could at the very least single out and display the fold change for a few glutamatergic marker genes.

Figure 2D. It is not clear whether the green channel in the image displays Gad1 RNA or eGFP fluorescence.

Figure 2L. Could the authors clarify where in the mesencephalon is the image taken?
Page 8, line 13. The authors should include here a reference for the Skor2GFP allele.
Figure 3Q,R. The morphology of the Skor2 KO cells appears changed (neurites are no longer prominent), however this possible morphological change is not mentioned in the manuscript. I wonder if this could be commented on (either confirmed or otherwise dismissed) in the text. This panel of the figure could also benefit from more explicit information on the developmental stage at which the image is taken (is this from the adult brain?).

Supplementary Figure 4. This figure would benefit from a more descriptive figure legend. It is an important figure, but not easy to navigate.

Figure 4. The authors could include a schematic presentation of the REM deprivation study, which should also display the circadian time at which animals are sacrificed for the histological analysis of cFos expression.

Figure 5C. A quantification that also includes the fraction of Ctb-labelled cells that are not Skor2 positive would be informative.

Related to Figure 6. It would be informative to know the fraction of Hcrtr positive neurons in the vLPAG/dMRF that are not Skort2 positive.

Significance

This manuscript makes a significant and novel contribution to the field of GABAergic neurogenesis in the midbrain. This is important because we know disproportionally less about the genetic and molecular control of the specification, differentiation and subtype diversity of inhibitory neurons in the midbrain than we do of the cortical ones. Thanks to studies such as this one, it is clear that radically different genetic programs for GABAergic neurons exist. It will be interesting to know how such diverse gene regulatory networks eventually result in unique functional properties versus the extent of functional convergence. This paper is impactful as it addresses the important and under investigated link between hardwired developmental programs and the functional neuronal networks that they contribute to generate.

Reviewer 3

Evidence, reproducibility and clarity

Below I make some specific comments:

Page 9, analysis of the CTb retrogradely labeled cells, a quantification should be reported in the text here in addition to the Sup Table. It is necessary to report the number of CTb and Cab/Skor2 neurons and the percentage of double-labeled cells (the use of many and frequently is not adapted). In addition, it is important to show the exact localization of the CTb injection site. It is indeed impossible to localize it with the photo shown. In the Figure illustrating the double-labeled cells, the cFos labeling is not really visible and should be improved.
How many mice and sections were used to analyzed Hcrt receptor colocalization? The description of the methods used is missing.
For the firing analysis, statistical analysis is necessary to separate the neurons in three subclasses.

Significance

The manuscript of Kirjavainen et al. is showing for the first time that a subpopulation of GABAergic neurons coexpressing Skor2 corresponds to the REM sleep off neurons gating REM sleep occurence by means of their projection to the SLD glutamatergic neurons. Their results are both new and extremely interesting and important. Indeed, there was no specific marker for these neurons and this was a key limitation to characterize them. Their results are very convincing and open the way to the identification of these neurons both neuroanatomically and functionnally.

Author response to reviewers' comments

Thank you for the careful review and considerate feedback to our manuscript.

We have now revised the manuscript according to your comments. We have attempted to address all the comments. Our responses and the changes to the manuscript are listed here, following each individual comment.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary:
The reticular formation of the midbrain contains numerous clusters of inhibitory (GABAergic) and excitatory (glutamatergic) neurons. These groups of neurons are functionally diverse, but their developmental history and molecular profile are poorly studied. Previously, Juha Partanen's group has demonstrated with a series of elegant mouse genetic studies that the transcription factor Gata2 is required for the development of all embryonic midbrain-derived GABAergic neurons. The study by Kirjavainen et al. now shows that Gata2 regulates a number of transcription factors expressed in specific GABAergic subpopulations in the mouse midbrain. This includes, among others, the

homeodomain transcription factor Nkx2-2 and the SKI family transcriptional repressor Skor2. Nkx2-2 and Skor2 are co-expressed in a small group of GABAergic precursor cells and both Gata2 and Nkx2-2 appear to be required for Skor2 expression. In the adult mouse and rat midbrain, the Skor2-positive GABAergic neurons are found at the boundary between the ventrolateral periaqueductal gray and the reticular formation of the midbrain. Since this very area contains REM-off neurons that regulate REM sleep, the authors investigate whether the Skor2-positive group contains these REM-off neurons. Indeed, c-FOS expression as an indicator of neuronal activity appears to be increased in Skor2-positive cells upon inhibition of REM sleep. Moreover, some Skor2-positive cells send projections to a pontine region associated with sleep control and they respond to orexins. These properties have also been found in REM-off neurons in the midbrain. In addition, the authors characterize the electrophysiological and morphological properties of Skor2-positive neurons and show that based on these characteristics, Skor2-positive neurons can be divided into several subgroups.

Major comments:
The majority of conclusions are well supported by the provided results. The following points would benefit from additional experiments, or the statements should be toned-down to reflect the experimental evidence:
1) Nkx2-2 knock-out: The authors conclude that Nkx2-2 is not required for cell survival of the neuronal population that would normally express Skor2
Cell death has only been tested at one developmental stage. Thus, it cannot be excluded that cell death occurs at later stages in the Nkx2-2 derived cells. Apoptosis could be assessed at additional developmental stages, or the conclusion could be toned down. The ideal experiment to exclude an effect on cell survival would be to cross the Skor2-GFP allele into the Nkx2-2 background. However, this would be a time and resource consuming experiment and is thus beyond the scope of the current study.

Author response: We show that in the Nkx2-2Cre/Cre mutants the Cre-labelled postmitotic precursors still exist, but do not express Skor2 (Fig 3 C,C', D, D'). However, we agree that the possibility of cell death at a later stage cannot be ruled out and we have removed the note about cell death.

2) Skor2 GFP/GFP mice, Figure 3M,M'. It appears that the knock-out contains more GFP positive cells, at least in the sections shown here. Have the authors investigated this? Quantification of cell numbers could be useful.

Author response: We have quantified the number of GFP positive cells in the dMRF/vlPAG region of Skor2GFP/+ and Skor2GFP/GFP embryos at E12.5 and found no difference between the genotypes. The results are shown in the Figure 3S and described in results, page 8.

3) Expression of Skor2 in cerebellar Purkinje cells: The image shown in the Figure is overexposed. The authors should provide a better image (appropriate exposure, higher magnification of the cerebellum) to support this statement.

Author response: The Purkinje cells show much higher level of GFP expression, as seen also in Figure 4H. We have now also added a Supplementary Figure (Supplementary Fig S5), demonstrating Skor2 protein and mRNA expression in the Purkinje cells of rat. The expression and role of Skor2 in Purkinje cells has been studied earlier in the mouse (Nakatani et al., 2014 PMID: 24491816 ; Wang et al., 2011 PMID: 21937600)

4) The authors claim that they „ followed the development of the neuronal precursors expressing Skor2 and Nkx2-2, asking whether they contribute to unique midbrain GABAergic nuclei in the postnatal brain"
Since the authors do not attempt fate-mapping of the Skor2 precursors this statement does not reflect the actual experiment. The authors analyze the Skor2-expressing population during embryogenesis and in the adult brain and assume that the Skor2-expressing precursors will develop into Skor2-expressing neurons. Moreover, it remains unclear whether the Skor2-expressing cells in the adult maintain Nkx2-2 expression. The statement should be re-phrased accordingly.

Author response: We rephrased the statement (page 8):“ Therefore, we asked whether Skor2; Nkx2-2 positive neurons are located in unique midbrain GABAergic nuclei in the postnatal brain”. We show that the Skor2 expressing neurons maintain Nkx2-2 expression in adult stage both in the mouse and the rat (Figure 2L-O, Figure 4O, R).

5) Abstract: "In the adult mouse as well as rat midbrain, the Nkx2-2 and Skor2 expressing GABAergic neurons locate at the boundary of the ventrolateral periaqueductal gray" and similar statements in the introduction (p.3) and results p.7/p.9
The authors do not show that the Skor2-positive cells express Nkx2-2 in the adult brain. The conclusion should be re-phrased accordingly.

Author response: Nkx2-2 and Skor2 co-expression in adult mouse midbrain GABAergic neurons is shown in Figure 2N-O. Images showing the co-expression of Nkx2-2 and Skor2 in the adult rat brain have been added (Figure 4 O-O’’’, R-R’’’).

6) Analysis of the co-expression of c-Fos and Skor2 revealed a significant increase in the proportion of c-Fos expressing dMRF/vlPAG Skor2+ neurons in the REM sleep deprived rats compared to the control groups or recovery group
The c-Fos expression is not convincing. c-Fos should fill out the nucleus, here it seems to be expressed in little speckles in the nucleus. The authors should provide better images and indicate with arrows which cells in these images they would consider positive.

Author response: We have updated the Figure 4 (Fig 4 M-R’’’) according to the reviewer’s suggestions.

7) Statement: Many of the CtB labelled cells showed co-expression of Skor2, suggesting that the Skor2+ dMRF/vlPAG neurons frequently project to the dorsolateral pons.
The results should be quantified (Skor2+/CTB+ and Skor2-/CTB+ cells) and the authors should indicate in the figure legend how many animals have been analyzed.

Author response: As the reviewer requests, we have quantified the co-localization of CtB and Skor2 in the area containing the Skor2 positive cells in the midbrain, and the results are now added (Results, p. 9-10). Our results indicate that in addition to the Skor2+ cells, the dMRF/vlPAG area also contains other cell types with equally robust connections to the dorsolateral pons. This is now also emphasized in the discussion (p.13)
The number of animals analysed are indicated in the results and now also in the figure legend (n=6).

Presentation of data and methods, replication of experiments and statistical analysis are appropriate.

Minor comments:

Figure 4: The TH and NF staining are not mentioned in the text. This should be added.

Author response: The reference to neurofilament staining and location of the Skor2+ cells in the mouse midbrain has now been added in Results, p. 8.

The methods describe that retrobreads were used for retrograde tracing, but this is not mentioned in the results or Figures. This should be removed.

Author response: The tracing experiments with retrobeads were indeed not included in this manuscript. We have removed the reference to retrobeads from the Methods.

p.10 The authors state that „The number of responding cells correlated with the proportion of the Orexin receptor expressing Skor2+ cells." It is not clear what is meant here - comparing numbers and proportion?

Author response: We detected Hcrtr1 and Hcrtr2 expression in ca 70% of the Skor2+ cells, and also the orexin evoked current was detected in ca 70% of the recorded cells. We hypothesize that the non-responding cells are the Hcrtr negative cells.

Sentence (p. 10) has been rephrased to: "The proportion of responding cells (Skor2^{+}: 32/44 cells, 72 %; Skor2^{-}: 0/14 cells; Fig. 6D) correlated with the proportion of the *Skor2*$^{+}$ cells expressing Orexin receptors." The threshold for Orexin-evoked response was set at the level of baseline RMS noise multiplied by two (added to Methods section). We hope this is now more clear.

The authors should highlight that Skor2 GFP/GFP mice die shortly after birth. Otherwise, the reader is left wondering why the authors did not explore the consequences of Skor2 inactivation on morphological and electrophysiological properties of the neurons or on REM sleep regulation.

Author response: We have modified the sentence on p. 13 to state this explicitly: "As the homozygous Skor2 mutant mice die shortly after birth (Nakatani et al., 2014), conditional inactivation of Skor2 in the midbrain will be needed to test the requirement of this TF for distinct subtype-specific anatomical and physiological properties of the dMRF/vlPAG GABAergic neurons."

Reviewer #1 (Significance (Required)):

The study by Kirjavainen et al. provides new insights into the developmental mechanisms that give rise to different groups of neurons in the ventral midbrain and a detailed characterization of a small group of GABAergic neurons expressing the transcription factor Skor2. Because little is known about the development of different GABAergic neurons in the midbrain, this is a valuable contribution to the field of neurodevelopmental biology. The authors also hypothesize that the Skor2-positive cells correspond to REM-off neurons. The present data support this hypothesis but do not prove it beyond doubt. For example, it remains an open question whether Skor2+ cells are identical to REM-off neurons, are a subset of REM-off neurons, or REM-off neurons are a subset of Skor2 neurons. If Skor2-expressing neurons are identical to REM-off neurons, Skor2 would be a valuable marker for further exploration of this neuron population and REM sleep regulation (e.g., by generating Skor2-Cre mice and using them for Skor2-specific tracing of neuronal projections and for functional manipulation). An analysis of the electrophysiological and morphological properties of Skor2-GFP neurons in a Skor2 conditional knockout model and an assessment of the functional consequences of Skor2 inactivation on the regulation of REM sleep would provide important insight into the functional significance of Skor2 in defining the functional properties of this neuronal population. Thus, this study lays the foundation for more detailed functional studies of this subset of GABAergic neurons in the midbrain.

Readership: neurodevelopmental researchers, researchers studying REM sleep, researchers studying the anatomical and functional diversity of neurons in the midbrain.

Reviewer, field of expertise: neurodevelopment, mouse genetics

Author response: We thank the reviewer for this nice summary. We agree with the comment suggesting further studies on Skor2 knockouts: while interesting, those would require extensive experiments that are beyond the scope of this manuscript. We now highlight this point in the manuscript, Discussion, p. 13-14: *"Recording and modulation of the activity of the Skor2 expressing neurons during sleep behavior will be needed to unambiguously show their involvement in REM sleep regulation. As the homozygous Skor2 mutant mice die shortly after birth, conditional inactivation of Skor2 in the midbrain will be needed to test the requirement of this TF for distinct subtype-specific anatomical and physiological properties of the dMRF/vlPAG GABAergic neurons."*.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this manuscript, Kaia Achim and Juha Partanen use cDNA arrays, progenitor labelling, conditional and constitutive mutagenesis and histological analysis to describe the development of a GABAergic neuronal subtype in the mouse midbrain that contributes to the ventrolateral periaqueductal gray and dorsomedial midbrain reticular formation (vlPAG/dMRF). This GABAergic subpopulation is defined by expression of Gata2, Nkx2.2 and Skor2 and the hierarchical regulation of these key transcription factors is also established. Using a classic (although with known limitations - see later)

REM sleep deprivation assay in rats and retrograde tract tracing, they find indirect evidence that suggests a potential role for this developmentally defined neuronal population in the control of NREM-REM sleep transitions. It is suggested that the Nkx2.2/Skor2 neurons coincide - at least in part - with the REM-off neurons that a well-known model of sleep regulation (see Saper, Fort, Luppi and others) describes as engaging in reciprocal and antagonistic interactions with REM-on neurons in the pontine region. Cell membrane electrical properties for the neurons are also described, revealing an apparent degree of functional heterogeneity.

The data in this manuscript are generally of high quality, clearly presented and, importantly, novel. The molecular and developmental part of the paper does not rise any major concerns. Similarly, I find the classification of the electrical and morphological properties of the cells on acute brain sections sufficiently detailed for a first description and conductive to further in vivo studies - however, I am not an expert in this area.

The analysis of cFos positive neurons in the REM sleep deprivation study is possibly the weakest part of the manuscript. The authors hint at some inconsistent staining for this marker and the immunofluorescence images in Fig. 4 M to R' rise some concerns as to the specificity of the labelling. A suggestion would be to provide data for cFos staining in other areas of the brain to frame the level of activation detected in the Skor2 population against (presumably) higher levels of activation observed elsewhere.

Author response: We agree that the resolution and quality of the original images showing cFos expression was not optimal. We have now included examples of the cFos and Skor2 stainings that better demonstrate their co-expression. In the experiments shown now, Nkx2-2 is included as an additional marker for these cells (Fig.4M-R’’’).

Although beyond the scope of this paper, if the authors would have the opportunity to repeat the REM sleep deprivation study, they could consider combining it with the retrograde tracing from the dorsal pontine region to measure the ratio of cFos/Ctb/Skor2 triple positive cells.

The method of the "inverted flower pot" that relies on the REM associated muscle atonia to achieve REM sleep deprivation, also causes a significative increase in stress and is unlikely to selectively disrupt REM sleep, given the very short duration of REM episodes in rodents. New methods have been developed which relay on real time, close circuit EEG/EMG detection and optogenetic interference with REM sleep to achieve more accurate and specific REM deprivation (see for instance work from the Adamantidis lab). While clearly such and advanced approach goes beyond the scope of the manuscript, it may be possible for the authors to assess whether other behavioural paradigms that also lead to increased stress and anxiety show a similar increase in activation of the target Skort2 population.

Author response: We agree that the “inverted flowerpot” method, although widely used in the earlier literature, can have effects on other aspects of brain function and behavior. We increase the specificity of the sleep-deprivation experiment by including the large-platform control group, but we agree that this may not completely alleviate this problem. Analysis of how stress and anxiety would affect the Skor2 expressing cells would be interesting. However, regardless of the outcome of these experiments, they likely would not provide definitive proof for the involvement of these cells in REM sleep regulation. To do this, experiments involving optogenetics, as described by the reviewer, would be needed. However, applying optogenetics methods would require establishment of completely new research tools for us, such as Skor2Cre mouse strain and, as also recognized by the reviewer, are beyond the scope of our manuscript. Our study describes developmental regulation and molecular properties of a specific midbrain GABAergic neuron subgroup and shows that they share characteristics of REM sleep regulatory neurons. As such, our study allows targeted approaches to study the biological functions of the Skor2-expressing cells.

We discuss the limitations of our study and possible other roles of these cells on p. 13.

Below are additional minor comments that, in my view, could help make the manuscript more accessible to a broader audience. In order of appearance in the manuscript.

Figure 1. It would be useful to see somewhere in the figure the schematic of the division of the midbrain neuroepithelium in "m" progenitor domains. This could be overlaid for instance on the image in C.

Author response: Domain borders and labels are now added on the panel D.

Figure 1B. it would be helpful to place an arrowhead also in L', similar to L.

Author response: Arrowheads are now added in L' and O'.

Table S1. Please explain the colour code used.

Author response: The colour code is explained on the right side of the table.

Table S2. Check the headings, as currently not readable.

Author response: The headings are now updated, using more descriptive terms.

Related to Figure 1 and Supplementary Figure 1. Please clarify the criteria for selection of candidate genes used in qRT-PCR and ISH validation.

Author response: As TFs were enriched among the down-regulated genes, we focused on them in our ISH experiments. For the qRT-PCR validation, we also used known GABAergic markers and genes with different fold-changes. This is now explained in the results (p. 5).

Related to Figure 1 and Supplementary Figure 1. Please comment on the results for Gata2. How is the discrepancy between fold change in RT-PCR and ISH/array explained?

Author response: The microarray probe for Gata2 is targeting exon 6 of the gene, which is outside of the floxed region of the Gata2 conditional allele. Thus, it is possible that truncated Gata2 gene product is present in the $Gata2^{cko}$, which however should not be functional as transcription factor as the floxed area in $Gata2^{flox}$ contains the DNA binding region of Gata2. This is now mentioned in the Results (p. 5) and legend of Supplementary Figure S1.

Related to Figure 1 and Supplementary Table 1. In view of the known role of Gata2 as lineage selector gene, I would have expected to see glutamatergic lineage genes upregulated in the cKO sample. Why are these not included in the table? The authors could at the very least single out and display the fold change for a few glutamatergic marker genes.

Author response: Upregulated genes were included in the Supplementary Table S2, tabs 1 (upregulated in the Gata2cko ventral midbrain) and 2 (upregulated in the Gata2cko dorsal midbrain). Compared to the down-regulated genes, the fold changes of the upregulated genes are relatively modest. This is perhaps expected, as the relative increase of glutamatergic precursors (ca. max 2-fold increase) is not as dramatic as the decrease of the GABAergic precursors (which are completely depleted) (Kala et al. 2009 PMID: 19088086). Vglut2, the definitive glutamatergic marker in the midbrain at this stage, was not found on the list of upregulated genes in the microarray analysis, despite the fact that it is clearly upregulated in the midbrain GABAergic regions in the Gata2cko mutants (Kala et al. 2009 PMID: 19088086).

Figure 2D. It is not clear whether the green channel in the image displays Gad1 RNA or eGFP fluorescence.

Author response: The image shows Gad1 RNA expression. The labeling has been changed to avoid confusion.

Figure 2L. Could the authors clarify where in the mesencephalon is the image taken?

Author response: The image shows the dMRF/vlPAG area in adult midbrain, corresponding to the region shown at the E18.5 in panels J, K and indicated with arrowhead. Explanation is now added to the Figure 2 legend (current Figure 2N-O).

Page 8, line 13. The authors should include here a reference for the Skor2GFP allele.

Author response: A reference is now added.

Figure 3Q,R. The morphology of the Skor2 KO cells appears changed (neurites are no longer prominent), however this possible morphological change is not mentioned in the manuscript. I wonder if this could be commented on (either confirmed or otherwise dismissed) in the text. This panel of the figure could also benefit from more explicit information on the developmental stage at which the image is taken (is this from the adult brain?).

Author response: We do not observe consistent differences in the morphology of Skor2GFP/+ and Skor2GFP/GFP cells. We have added a supplementary figure (Supplementary Figure S4) showing the morphology of GFP+ cells in both genotypes, and mentioned in the Results, page 8. The stage (E18.5) is now added in the Figure 3 and is reported in the Figure legend.

Supplementary Figure 4. This figure would benefit from a more descriptive figure legend. It is an important figure, but not easy to navigate.

Author response: The legend of the Supplementary Figure 4 (current Supplementary Figure S6) has been modified. We hope it is now more explanatory.

Figure 4. The authors could include a schematic presentation of the REM deprivation study, which should also display the circadian time at which animals are sacrificed for the histological analysis of cFos expression.

Author response: We have now updated the Figure 4, adding a schematic presentation of the flowerpot assay for REM sleep deprivation (Fig. 4K).

Figure 5C. A quantification that also includes the fraction of Ctb-labelled cells that are not Skor2 positive would be informative.

Author response: We have counted the CtB+ cells in the dMRF/vlPAG region and analysed the proportion of CtB label incorporation in the Skor2 positive and Skor2 negative cells in the dMRF/vlPAG (Results p. 9-10).

Related to Figure 6. It would be informative to know the fraction of Hcrtr positive neurons in the vLPAG/dMRF that are not Skor2 positive.

Author response: We have quantified the Hcrtr1+, Skor2 (+/-) and Hcrtr2+, Skor2 (+/-) cells in the dMRF/vlPAG region containing the Skor2 expressing cells. About 25% of the Skor2 negative cells and 70% of the Skor2 positive cells in dMRF/vlPAG express Orexin receptors. The quantification results are summarized in the Figure 6C-D.

Reviewer #2 (Significance (Required)):

This manuscript makes a significant and novel contribution to the field of GABAergic neurogenesis in the midbrain. This is important because we know disproportionally less about the genetic and molecular control of the specification, differentiation and subtype diversity of inhibitory neurons in the midbrain than we do of the cortical ones. Thanks to studies such as this one, it is clear that radically different genetic programs for GABAergic neurons exist. It will be interesting to know how such diverse gene regulatory networks eventually result in unique functional properties versus the extent of functional convergence. This paper is impactful as it addresses the important and under investigated link between hardwired developmental programs and the functional neuronal networks that they contribute to generate.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Below I make some specific comments:

Page 9, analysis of the CTb retrogradely labeled cells, a quantification should be reported in the text here in addition to the Sup Table. It is necessary to report the number of CTb and Cab/Skor2 neurons and the percentage of double-labeled cells (the use of many and frequently is not adapted). In addition, it is important to show the exact localization of the CTb injection site. It is indeed impossible to localize it with the photo shown. In the Figure illustrating the double-labeled cells, the cFos labeling is not really visible and should be improved.

Author response: We have quantified CtB incorporation in the cells of dMRF/vlPAG region and analysed how many of the Skor2 positive and how many of the Skor2 negative cells are labelled by CtB (Results p.9-10). As mentioned in the results and emphasized in the discussion (p.13), other cell types in the dMRF/vlPAG, besides the Skor2^{+} cells, show projections to the dorsolateral pons.
We have replaced the images of cFos expression (Fig 4M-R'''').
The coordinates of the CtB injection are given in the Materials and Methods.

How many mice and sections were used to analyzed Hcrt receptor colocalization? The description of the methods used is missing.

Author response: The number of Skor2$^{GFP/+}$ animals (n=4) used for the Hcrtr receptor colocalization quantification is now mentioned (p. 9). The IHC method and antibodies are described in the materials and methods.

For the firing analysis, statistical analysis is necessary to separate the neurons in three subclasses.

Author response: The placement of neurons into subclasses was done based on the firing pattern (stuttering or adapting) and the shape of AHP (one component- or two components AHP). This was done by classification of AP traces and confirmed afterwards by comparing the individual properties of AP firing (Figure 7, Supplementary Figure S7, p.10). The detailed description of classification procedure is now included to the "Results" section. As shown in Supplementary Fig. S7, the three subgroups of Skor2 expressing neurons are similar in several other electrophysiological properties. Our results demonstrate heterogeneity of these neurons, not necessarily discrete subclasses, although we use the term "subgroup" to illustrate the heterogeneity.

Reviewer #3 (Significance (Required)):

The manuscript of Kirjavainen et al. is showing for the first time that a subpopulation of GABAergic neurons coexpressing Skor2 corresponds to the REM sleep off neurons gating REM sleep occurrence by means of their projection to the SLD glutamatergic neurons. Their results are both new and extremely interesting and important. Indeed, there was no specific marker for these neurons and this was a key limitation to characterize them. Their results are very convincing and open the way to the identification of these neurons both neuroanatomically and functionally.

Original submission

First decision letter

MS ID#: DEVELOP/2022/200937

MS TITLE: Gata2, Nkx2-2 and Skor2 form a transcription factor network regulating development of a midbrain GABAergic neuron subtype with characteristics of REM sleep regulatory neurons

AUTHORS: Kaia Achim, Anna Kirjavainen, Parul Singh, Laura Lahti, Patricia Seja, Zoltan Lelkes, Aki Makkonen, Sami Kilpinen, Yuichi Ono, Marjo Salminen, Tarja Stenberg, Svetlana Molchanova, Juha M Partanen, and Teemu Aitta-Aho
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The study by Kirjavainen et al. provides new and important insights into the developmental mechanisms that give rise to different groups of neurons in the ventral midbrain and a detailed characterization of a small group of GABAergic neurons expressing the transcription factor Skor2. Little is known about the development of different GABAergic neurons in the midbrain, thus this is a very valuable contribution to the field of neurodevelopmental biology.

Comments for the author

The authors have addressed all my previous comments and I have no further suggestions.

Reviewer 2

Advance summary and potential significance to field

This manuscript makes a significant and novel contribution to the field of developmental neurobiology. It provides novel data on early molecular control of lineage fate decisions and follows on to show specific functional roles of such developmentally defined cells in the adult brain. Furthermore, the paper expands our currently limited understanding of GABAergic neurogenesis in the midbrain. This is important because, while we know disproportionally less about the genetic and molecular control of GABAergic neurogenesis in the midbrain compared to telencephalic one, all evidence points toward the existence of a different developmental logic to make GABAergic neurons in the midbrain. The data presented in this manuscript provides new genetic markers to specifically target and manipulate pontine neurons involved in the regulation of rapid eye movement (REM) sleep.

Comments for the author

This manuscript was initially submitted to Review Commons and I provided my review and feedback to the authors at the time. The manuscript has now been submitted for publication upon through revision. This report will take into account the authors' reply to my initial review and the action they have taken to address any pending issues.

The authors have addressed each one of the specific comments that I had raised. The revised analysis of the cFos expression upon REM sleep deprivation is now much improved and the immunohistochemistry signal unambiguous. New quantifications of the fraction of Skor2-expressing neurons that also express Orexin receptors now strengthen the characterisation of the adult Skor2 population.

I support publication of the revised manuscript in Development.