Multimodal cell lineage reconstruction in the hindbrain reveals a link between progenitor origin and activity patterning

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ABSTRACT

The vertebrate brain develops through a precisely orchestrated sequence of events, where neural progenitors generate neuronal diversity. However, the importance of stem cell origin in the acquisition of functional characteristics and the assembly into circuits are largely unknown. Here, by multimodal cell lineage reconstruction we reveal a link between progenitor origin and excitatory and inhibitory activity in the hindbrain. By combining progenitor intersectional fate mapping with newly developed computational tools and CRISPR/Cas9-based loss-of-function experiments, we discovered the differential temporal contribution of distinct progenitor pools –defined by proneural gene expression– to both glutamatergic and GABAergic neurons. Whereas *neurog1*-progenitors contribute to both lineages in early embryonic stages, later, other progenitor pools either also assume this role or take over. Using calcium imaging in combination with genetic perturbations, we unveiled that spatiotemporally defined stem cell origin determines specific neuronal activity features. Overall, we provide in vivo evidence for a link between cell ontogeny and functionality in the vertebrate hindbrain.

MAIN

The survival of an organism requires the coordination of the activities of its organs and of these with its environment. The responsibility for these tasks falls on the neuronal circuits, which are generated during embryonic development. Population activity in developing circuits critically reflects the structural and functional organization of the emerging network and can itself influence the developmental process[1,2]. While much is known about how neurons are generated[3], the variables that drive a seemingly uniform pool of neural progenitor cells into the specific neuronal types in a temporally coordinated manner to build the complex networks of a functional brain are still not clear.

The embryonic brainstem —the hindbrain— is a good model to tackle how neuronal diversity is generated because it occupies only 11% of the brain mass yet harbors 80% of the total brain neurons[4]. The hindbrain is highly conserved along evolution[5] and the whole array of neurons is produced extremely rapidly at early embryonic stages, since the progenitor pools are depleted in the adult[6,7]. These neurons establish early functional circuits, which are essential for fundamental physiological responses/behaviors, such as respiration, circulation, arousal and motor coordination[8–10]. Thus, the hindbrain is uniquely suited to study how temporal control and progenitor competence are coupled.

During embryonic development, several cell types are known to originate from more than one source. However, how progenitor origin influences the production of functional neuronal subtypes is still an open question. During central nervous system development, cell proliferation and differentiation must be tightly coordinated —early on, proliferation dominates, allowing the tissue to grow; later, more cells differentiate and exit the cell cycle, and the growth rate approaches zero[11,12]. Spatial cues regulating cell fate decisions have been established, and it is now known that temporal mechanisms also play a large role in controlling progenitor competence. For instance, in the mammalian cortex, genes specifically expressed in early- or late-state progenitors can have dynamic inverted temporal gradients, as shown by transcriptomic analyses[13], and neural progenitors gradually switch from specifying earlier-born cell fates to specifying later-born neurons, as shown by transplantation experiments[13–15]. In the hindbrain, the spatial distribution of neurons in the tissue foreshadows the time of neuronal differentiation, with an inner–outer gradient[7] in which cells born first are more innerly located than cells born last. Thus, these observations raise the question whether both

progenitor origin and order of neuronal differentiation confer specific neuronal activity patterns. Addressing how this occurs has been technically challenging to date since it is difficult to trace the origin of functional neurons while recording their activity. However, the zebrafish hindbrain offers excellent experimental suitability since its transparency and small size allow high-resolution live imaging and genetic lineage tracing at whole-organ scale, while genome editing enables precise perturbations.

Here, we address the role of the spatiotemporally defined progenitor origin in the construction of excitatory (glutamatergic) and inhibitory (GABAergic) networks leading to the emergence of spontaneous neuronal activity. By combining progenitor intersectional fate mapping[16] and CRISPR-based perturbation experiments with a new computational pipeline to generate 3D reference maps of whole brains, we establish the differential temporal contribution of distinct progenitor pools –defined by proneural gene expression– to the glutamatergic and GABAergic lineages. Whereas *neurog1*-progenitors contribute to both lineages in early embryonic stages, later, other progenitor pools either also assume this role (for excitatory neurons), or take over (for inhibitory neurons). Further, by using optogenetic sensors to record calcium activity and 3D spatial correlation in the entire developing circuit we unveil that cell lineage origin is crucial in defining neuronal activity. Finally, by performing calcium sensor recordings upon perturbation of distinct progenitor pools we demonstrate that temporally defined stem cell origin determines specific functional neuronal features: early-born, medially located stem cells produce neurons characterized by high-amplitude, low-frequency, and synchronized activity, likely establishing the baseline circuit architecture; by contrast, later-born, laterally located stem cells produce neurons with low-amplitude, high-frequency, and asynchronous activity, suggesting a role in more specific fine-tuning. Thus, both progenitor origin and age are crucial parameters in establishing terminal characteristics, such as the neurotransmitter identity and function, demonstrating that the lineage and developmental trajectory of a cell are key determinants of cellular functional identity.

RESULTS

To reveal how spatial neurogenesis patterns are established in the hindbrain, we first assessed the temporal expression of proneural genes (PN) –transcription factors that commit progenitors towards the neuronal lineage– in the neural progenitor domain. At 24hpf, their expression differed along the dorsoventral (DV) axis (Figure 1a–e), with 5 distinct progenitor pools defined according to the PN-gene combinations. While most PN-gene allocation into the progenitor pools was similar to that of the spinal cord[17,18], the most dorsal *neurog1* expression domain was absent (Figure 1a–a'). *neurog1*-expressing progenitors were mainly, but not exclusively, contained within the *ascl1b*-domain (Figure 1a–b, e), whereas *atoh1a* and *ptf1a* progenitors were segregated to the dorsal-most part of the tissue with no observable overlap with other PN genes (Figure 1c–e; [19]). Progenitors placed in the dorsal domain at 24hpf were located dorsolateral at 48hpf due to the progressive opening of the neural tube (Figure 1f–j). By 48hpf, most of the *neurog1*-domain was still contained within the *ascl1b*-region (Figure 1f–g, j), which expanded laterally towards the *ptf1a*-domain (Figure 1g–h, j). This change in the temporal PN expression profile may result in part from two intertwined morphogenetic events that occur during development, the growth of the neuronal differentiation domain at the expense of the progenitor territory[7,20] and the formation of the brain lumen[19–21]. The expression of PN genes along the anteroposterior (AP) axis differed (Extended Data Figure 1a–j). At 24hpf, *ascl1b* was expressed all along the AP axis in a ventral domain comprising *neurog1*, and only in the posterior hindbrain in a specific dorsal domain comprised between the *ptf1a* and *atoh1a* domains (Extended Data Figure 1 a–b, e; a'–b', e'). By 48hpf, this dorsally located *ascl1b* domain displayed *neurog1* as well, while still being absent in more anterior regions (Figure 1f, I; f', I'). Overall, these results show that PN genes progressively pattern the neural progenitor domain (Figure 1k).

Next, to monitor the growth of the *neurog1*-progenitor pool we built up the Tg[*neurog1*:Cre-P2a-nlsCerulean] transgenic zebrafish line, with Cre under the control of *neurog1* promoter. In transgenic embryos, *neurog1* mRNA expression was restricted to the progenitor domain (Extended Data Figure 2a–c, a'–c'), and accordingly, HCR analysis revealed overlapping expression of *Cre* and *neurog1* (Extended Data Figure 2d–d'', e–e''). *In vivo* Light Sheet Fluorescence Microscopy (LSFM) imaging of Cerulean expression demonstrated this new transgenic line faithfully recapitulates the expected neurog1 spatiotemporal patterning during

hindbrain morphogenesis (Extended Data Figure 2f–j, f'–j'), bringing further evidence that indeed it was a good readout of *neurog1*-expressing stem cells.

neurog1-progenitors differentially contribute to excitatory and inhibitory neurons with distinct temporal dynamics

We explored the differential temporal contribution of distinct PN-expressing progenitor pools to the excitatory (glutamatergic) and inhibitory (GABAergic) neuronal circuits (Figure 1l), which constitute 80% of the overall hindbrain neurons born before 72hpf[20]. We used progenitor-restricted intersectional mapping[16] to singularize cell lineages arising from specific progenitor populations and assessed their temporal contribution to the differentiated neuronal derivatives (Figure 1l).

To address the contribution of *neurog1*-progenitors to the specific glutamatergic (*vglut2a*) or GABAergic (*gad1b*) neuronal subsets, Tg[*neurog1*:Cre-P2a-nlsCerulean] fish (hereafter Tg[*neurog1*:Cre]) were crossed with Tg[*vglut2a/gad1b*:Lox-DsRed-Lox-GFP] fish[22] (hereafter Tg[*vglut2a/gad1b*:switch]) and the resulting embryos were *in vivo* imaged during periods encompassing the beginning of neuronal differentiation (24hpf) until most of the hindbrain cells are postmitotic neurons (72hpf)[7,20]. The production of glutamatergic and GABAergic neurons followed distinct dynamics with several progenitor pools contributing to their growth (Figure 1m–o, p–r; see *neurog1*-derived cells in green and the ones derived from other progenitors in magenta). *neurog1*-progenitors generated both types of differentiated neurons; while glutamatergic derivatives were already widely present in the hindbrain by 24hpf (see green cells in Figure 1m, m'), at that time the hindbrain was almost devoid of GABAergic neurons and most of the those derived from *neurog1*-cells corresponded to sensory ganglia outside the hindbrain[23,24] (see asterisk in Figure 1p, p'). By 48hpf, although *neurog1*-progenitors were still contributing to glutamatergic neurons, other PN-expressing progenitors started contributing to them (see magenta cells in Figure 1n, n') and this was maintained by 72hpf (Figure 1o, o'). On the other hand, by 48hpf most of GABAergic neurons derived from *neurog1*-progenitors (see green cells in Figure 1q, q'), and by 72hpf, GABAergic neurons were contributed strongly by other progenitor pools (see magenta cells in Figure 1r, r'). To further address the progenitor contribution dynamics, we performed LSFM recordings and observed two distinct behaviors for these neurotransmitter's defined subsets. Glutamatergic neurons were simultaneously and consistently produced by both *neurog1*-

expressing progenitors and other pools across development, whereas GABAergic neurons were exclusively produced by *neurog1*-progenitors early on, and later they were generated by other contributors (Extended Data Videos 1–4). Although the secondary production of GABAergic neurons was initiated later, it resulted in the production of a relatively similar proportion of neurons (Extended Data Videos 3–4). These results demonstrate a differential temporal growth of glutamatergic and GABAergic hindbrain neurons, with distinct spatiotemporal contribution of progenitor pools. Whereas *neurog1*-progenitors contribute to both lineages in early embryonic stages, later, other progenitor pools either also assume this role (for excitatory neurons), or take over (for inhibitory neurons). Next, we wanted to identify the other stem cell pools contributing to these lineages.

Glutamatergic and GABAergic lineages derive from distinct progenitor pools combinations

Thus, to globally resolve the heterogeneity of contributors and identify them, we combined progenitor-restricted intersectional mapping using the Tg[*neurog1*:Cre] and the Tg[*vglut2a*/*gad1b*:switch] lines with a CRISPR/Cas9-based approach that produces loss-of-function (LOF) phenotypes in F_0 by redundantly targeting single genes[25,26] –and therefore impair the formation of specific progenitor pools. Either *neurog1*, *ascl1b*, *ptf1a* or *atoh1a* proneural genes were targeted by injecting one cell stage embryos with four different gRNA per proneural gene, and we assessed the impact *i)* on the expression of PN genes in the adjacent domains by HCR-multiplex transcript analysis (Extended Figure Data 3; Figure 2a) and *ii)* on the glutamatergic and GABAergic lineages (Figure 2b). *neurog1*_gRNA injections resulted in smaller hindbrains with no dramatic changes in adjacent PN gene expression (Extended Data Figure 3d–e, i–j), although there was an upregulation of *neurog1* in the statoacoustic ganglion as previously reported (data not shown, [23,24]). *ascl1b*_gRNA injected embryos displayed smaller hindbrains with a smaller *neurog1* territory (Extended Data Figure 3d, f, i, k) suggesting a regulation of *neurog1* by *ascl1b*. *ptf1a*_gRNA resulted in an expansion of *ascl1b* (Extended Data Figure 3d, g, i, l) indicating that *ptf1a- and ascl1b*-domains interact. Finally, *atoh1a*_gRNA did not dramatically impact the adjacent *ptf1a* domain (Extended Data Figure 3d, h, i, m) showing that there is no cross-regulation as previously demonstrated in the *atoh1a*^{fh282} mutants[19].

Next, we analyzed the impact of proneural gene LOF on the glutamatergic and GABAergic lineages by 72hpf (Figure 2b). Tg[neurog1:Cre:*vglut2a*/*gad1b*:switch] embryos were injected

with control or PN_gRNAs, imaged at 72hpf, and cell lineages' analyses were performed to *i)* quantify the proportions of glutamatergic and GABAergic neurons derived from the different progenitor populations and *ii)* construct spatial distribution models allowing the evaluation of the impact of LOF on the neuronal populations across the tissue (Figure 2b). To quantify the outcome on the neuronal populations we established a computational workflow combining *i)* a newly generated deep learning-based software, RAD (Rigid Alignment with Deep learning) to perform rigid registration among samples, which enabled supervised or unsupervised alignment of tissues using a trained digital neural network (see Github), *ii)* the latest version of Cellpose, a deep learning single cell segmentation framework, and *iii)* a set of Python scripts to quantify, process and generate representative 3D-models averaging 10 hindbrains per condition including different transgenic backgrounds (Figure 2b). Our averaged 3D-models (Figure 2c–f) revealed no spatially defined differences within the glutamatergic population upon *ascl1b* and *ptf1a* LOF (Figure 2c–e), but an important decrease upon *atoh1a* downregulation (Figure 2c, f). Quantification of individual samples showed that the global glutamatergic population was significantly reduced upon *ascl1b* and *atoh1a* LOF and that this reduction was mostly due to a cell population located in the anterior hindbrain (r1-r5) (Figure 2h). A minor reduction was also observed anteriorly upon *ptf1a* LOF, demonstrating an indirect effect of the GABAergic perturbation. The shift in the proportion of *neurog1*-derivatives in the r1-r5 region placed *atoh1a*-progenitors as the major other contributor –besides *neurog1*-progenitors– to the glutamatergic pool (Figure 2i). To spatially map these progenitors, we put forward the depleted and compensated cell subsets across the hindbrain by performing deltas of spatial distribution between control and loss-of-function 3D-models (Extended Data Figure 4a–d). By combining the depleted subset of the various loss-of-function experiments we generated a 3D global model of PN-progenitor requirements for glutamatergic neurons across the hindbrain, which informed us about the spatial requirements for *neurog1*-, *ascl1b*- and *atoh1a*-progenitors within the final neuronal population (Figure 2g). These results indicate that the several progenitor pools that contribute to glutamatergic neurons are not segregated progenitor subsets, except for *atoh1a*-progenitors, indispensable to the dorsolateral neurons (Figure 2g). When non-*neurog1* derivatives were overlapped with the proneural gene requirements' map, the quantifications revealed that this population was significantly enriched in the *atoh1a*-dependent subset, bringing further confirmation that *atoh1a* was the other major contributor to the glutamatergic neuronal population (Figure 2j).

When the same strategy was applied to the GABAergic neurons, we observed a dramatic decrease of the GABAergic population upon downregulation of *ptf1a* (Figure 2k–n). Quantifications revealed a significant reduction of GABA neurons upon both *neurog1* and *ptf1a* LOF (Figure 2p), which was restricted to the anterior hindbrain in the case of *neurog1*_gRNA but was evenly distributed along the AP axis in the context of the *ptf1a*_gRNA. To further confirm that the *neurog1*-population was able to produce GABAergic neurons independently of *ptf1a*, we performed LSFM imaging in the cell lineage tracing reporter background and observed that indeed *neurog1*-cells were giving rise to GABAergic neurons in the absence of *ptf1a* (Extended Data Video 5). Moreover, we revealed a decrease in the ratio of *neurog1*-derived neurons upon *ascl1b* LOF in the whole hindbrain with no significant impact on the global GABAergic cell quantity (Figure 2q) unveiling a compensation mechanism and suggesting that at least a subset of the *neurog1*-progenitors is dependent on *ascl1b*. When the progenitors' origin requirement maps were confronted to our lineage tracing models, we further confirmed *ptf1a* to be the most likely major other contributor to the GABAergic lineage (Figure 2o; see non-overlayed 3D-maps in Extended Data Figure 4j). We plotted the depleted and compensated cell subsets across the hindbrain by performing deltas of spatial distribution between control and loss-of-function 3D-models (Extended Data Figure 4e–i). These results back up our previous observation that *neurog1*-progenitors are the main contributors to GABAergic neurons at early stages, and that later, *ptf1a*-progenitors take the relay. Accordingly, the *neurog1*-expression domain located laterally, between the *atoh1a* and the *ptf1a* domains, disappeared upon *ascl1b* or *ptf1a* LOF, as shown by HCR experiments at 48hpf (see asterisk in Extended Figure Data 3i, k–m). Overall, these observations suggest that the GABAergic neurons originating from *neurog1*-progenitors derive from two cell populations, one located medially that is *ptf1a*-independent and one located laterally that is *ptf1a* and *ascl1b* dependent.

Progenitor origin defines early functional patterning of hindbrain neurons

To understand the extent to which the ontogeny of a cell is linked to its function, we first addressed the composition of functional cell populations in the embryonic hindbrain at 72hpf. Since at this stage no neuronal activity recordings were publicly available, we made use of the Tg[*elavl3*:jrGECO1b] transgenic line to record spontaneous activity in the whole hindbrain (Figure 3a; Extended Data Figure 5a) using high-speed simultaneous multi-view light-sheet

microscopy (hs-SiMView[27]). To extract single cell information, we developed an image analysis pipeline to establish individual cell profiles of the detectable active neurons across the whole hindbrain (Figure 3a; Extended Data Figure 5b). We identified 4 communities using Louvain clustering for correlating the cell-to-cell activity (Figure 3b), which upon UMAP analysis plotted in a segregated manner (Figure 3c). Different neuronal communities displayed specific features (Figure 3d). For instance, each community displayed a different synchronization score, community 4 being by far the less synchronized (Figure 3d; C1 = 0.66, C2 = 0.52, C3 = 0.49, C4 = 0.06). To explore whether neurons and communities displaying different activity patterns were differentially located, we plotted their spatial distribution and revealed that indeed neuronal activity features were specifically allocated within the hindbrain (Figure 3e–g). For instance, C4 was the most laterally located community with a particular lack of synchronicity combined with a high number of low amplitude calcium spikes (Figure 3d, g). To seek whether there was a correlation between neuronal activity and cell lineage, we used whole registered zebrafish hindbrain images and compared distinct neuronal differentiation patterns in a unified spatiotemporal reference space[7] (see Methods). This provided a platform to dissect the relationship between structure, gene expression and function in brain networks (Figure 3a). Dissecting the neuronal composition of these communities revealed no cell lineage exclusivity (Figure 3h), hinting at indispensable circuit scale properties of the analyzed derivatives. Nevertheless, there was an enrichment of given lineages in communities displaying widely different activity patterns. When dissecting the composition of communities within the glutamatergic and GABAergic populations deriving from *neurog1*- or non-*neurog1*-progenitors we observed noticeable differences in community configuration. The *neurog1*-derived neurons constituted most of both glutamatergic and GABAergic active populations (Figure 3h; see green and cyan bars in C1–C4; Extended Data Figure 5f, h). Notably, community 2 displaying the highest count of synchronized spikes was enriched in *neurog1*-derived glutamatergic neurons (Figure 3d, see green bars in 3h; Extended Data Figure 5f), and community 3 showing lower count but higher intensity spiking was enriched in *neurog1*-derived GABAergic neurons (Figure 3d, see cyan bars in 3h; Extended Data Figure 5h). On the other hand, community 4 displaying the most variable and least synchronized activity presented the highest proportion of non-*neurog1* derivatives (Figure 3d, see magenta and red bars in 3h; Extended Data Figure 5g, i). Most of the active neurons in all communities were glutamatergic and GABAergic neurons, although there was a small proportion of other

neuronal type (Figure 3h, see grey bars). To further identify the potential lineage drivers of the observed functional features we analyzed the cell lineage requirements of the different communities (Figure 3i). We observed a significant enrichment in the proportion of *atoh1a*-dependent glutamatergic and *ptf1a*-dependent GABAergic neurons attributed to community 4 (Figure 3i; see magenta and blue bars in C4). This observation aligned with our progenitor contribution assessment, which associated community 4 with non-*neurog1* derivatives.
To confirm the association of the *neurog1*-derived neuronal subset to a given synchronicity profile we first analyzed the individual lineage-associated subset community compositions and observed that non-*neurog1* derived GABA and glutamatergic populations systematically contained more cells in the unsynchronized community 4 (see C4 in Extended Data Figure 5f–i, with community counts in bold and a black asterisk indicating the corresponding cell-cell synchronicity matrice). Taken together, those distributions suggest a particular progenitor cell origin to the be the source of the observed synchronicity. To prove this, we measured the cell-cell synchronicity scores across the lineage defined derivatives and observed significant differences between *neurog1* and non-*neurog1* derivatives, suggesting *neurog1* derivatives to be specifically associated with synchronized activity (Figure 3h). Finally, after associating a circuit feature to *neurog1*-derivatives we wanted to evaluate if a given lineage could be associated with specific single cell signal features. Although some functional features such as the global synchronicity score relied on the cell lineage (Figure 3j), not all activity features did so (Figure 3k). When we measured spike counts across the recording, we noticed that the glutamatergic neurons dependent on *atoh1a*-progenitors displayed a significant increase in spike count compared to the *ascl1b*-dependent ones, but no differences were observed in GABAergic neurons (Figure 3k). Taken together these results demonstrate differences of activity among the lineages indicating a correlation between the lineage and both circuit and signal scale activity features.

To further demonstrate causality between the presence of cell lineage specific populations and the activity patterns observed across the hindbrain, we combined calcium imaging recordings with the CRISPR/Cas9-based loss-of-function approach. We observed major changes in neuronal activity (Figure 4a–e; Extended Data Videos 6–10) such as a decrease in cell-cell activity correlations (i.e synchronicity) upon *ascl1b* loss of function (Figure 4a–c, a’–c’). This was further confirmed by the analysis of the average activity pattern (Figure 4a’’–c’’),

in which *neurog1*_gRNA embryos displayed an overall decrease in signal amplitude with no loss of synchronicity (compare Figure 4a'' and 4b''). *ascl1b*_gRNA embryos showed the highest standard deviation and lack of temporally defined spiking (compare Figure 4a'' and 4c''). Since *ascl1b* and *neurog1* were expressed in similar domains (Extended Data Figure 3), these results suggest that the synchronicity enrichment in *neurog1*-derived populations depend on *ascl1b*. Neuronal activity upon *ptf1a* loss-of-function resulted in wider spikes without losing circuit-wide synchronicity or amplitude of signal (compare Figure 4a'' and 4d''), with the possibility that the generation of spiking trails could be associated to the direct disruption of spike timings resulting from an imbalanced inhibition[28]. On the contrary, upon *atoh1a* loss-of-function we observed no loss of synchronicity or amplitude of signal, whereas the number of spikes was greatly increased across the whole tissue, although not in the form of spiking trails but as individual events (compare Figure 4a'' and 4e''). Since the *atoh1a*-dependant glutamatergic neurons displaying significantly high spike count constituted the major component of the asynchronous community (C4, Figure 3i), and localized proximally sensory neurons, it is likely that *atoh1a*-derived glutamatergic neurons are involved in decoding the sensory input in the hindbrain.

DISCUSSION

Our research highlights that excitatory and inhibitory neuron originate from different progenitor pools –defined according to proneural gene expression– and provides *in vivo* evidence for a link between neuronal cell ontogeny and function. Moreover, our findings reveal that the spatiotemporal order of neuronal differentiation is essential to establish functional neuronal circuits (Figure 5). The prevailing view is that neuronal identity is defined by specific gene expression programs, which are executed by gene regulatory networks[29]. However, we believe that this is an incomplete depiction of the process, as *i*) lineage history within a given tissue context and temporal progression are emerging as central to defining cell fate; and *ii*) main drivers of neuronal differentiation are not enough to generate neurons with distinct morphologies and functional regional localizations in the central nervous system[30,31]. Our results challenge the prevailing view in vertebrate brain neurogenesis that both excitatory neurons and inhibitory neurons derive from two spatially segregated progenitors that express either *neurog1* (excitatory glutamatergic neurons) or *ptf1* (inhibitory GABAergic neurons)[32,33]. We demonstrate that they instead arise from several progenitor pools. Glutamatergic neurons derive mainly from *neurog1*, *ascl1b* and *atoh1a* progenitors whereas GABAergic neurons derive mainly from *neurog1* and *ptf1a* progenitors. The multiple and distinct sources of glutamatergic and GABAergic neuronal populations suggest the presence of lineage defined subcategories within those populations, as it has been demonstrated in other developmental systems such as the lymphatic vessels[34]. This indicates a certain plasticity of the stem cell pools during development, to maintain the robustness of the system. We further elucidate a crucial temporal component: at early embryonic stages, *neurog1*-progenitors contribute to both lineages; in later stages, other progenitor pools either take over (for inhibitory neurons) or also assume this role (for excitatory neurons). Several works suggested the importance of neuronal age-related patterning in forming a foundation for the construction of the networks underlying the many behaviors produced by the hindbrain[7,35,36]. Now, we provide a clearer picture as to the lineage spatial and temporal drivers leading to the generation of neuronal diversity in the hindbrain, supporting the notion that cell lineage and developmental trajectory are key determinants of cellular identity. Further, we provide *in vivo* models that integrate *temporal logic* within intact tissue architecture and help us to better interrogate brain development and to pave the way for cell therapies.

The combination of 3D- and *in vivo* experiments with new computational resources allowed us to have a common framework to globally interrogate the system and address previously inaccessible questions. In the future, combining this 3D lineage and activity map strategy with spatial transcriptomics or proteomics could further unveil the differential mechanisms underlying the formation of specific functional traits.

Although the emergence of new and increasingly sophisticated behaviors after birth is accompanied by dramatic increase of newly established synaptic connections in the nervous system. Here we provide the first evidence at the global tissue scale that the cell lineage is pivotal for encoding neuronal function early on, helping to explain how nascent connections are organized to build up new behaviors on top of existing functional circuits. The high reproducibility of neuronal activity patterns indicates the robustness of zebrafish embryos fundamental circuits for survival, in line with other studies showing that functional maturation of neurons is stereotyped, based on birth time and anatomical origin[37]. This does not mean that the early acquired functional features will be maintained up to adulthood; rather, it primarily tells us that neuronal activities crucial for life-sustaining of the individual as those coordinated by the hindbrain are established very early. One of these examples would be the hindbrain V2a neurons, which are necessary for spontaneously occurring swimming as early as 3dpf and display region-specific differences in the ability to elicit or stop swimming (anterior regions being less efficient)[38]. Therefore, we propose a model in which lineage not only encodes neurotransmitter identity but provides functional purpose to neuronal populations. This would be synchronizing for the most medial cells as shown upon disruption of *ascl1b*-progenitors, buffering for the neurons in between since upon LOF of *ptf1a* we have less frequent spikes and GABA has been shown to control action potential firing[28,39], and decoding for cells in the most lateral domain receiving the sensory inputs. This suggests that the orchestrated spatiotemporal patterning of proneural defined progenitors establishes neuronal diversity and circuit function allowing the essential hindbrain resilience and adaptation necessary for its life sustaining functions.

ONLINE METHODS

Ethics declarations and approval for animal experiments

All procedures were approved by the institutional animal care, the PRBB Ethics Committee in Animal Experimentation and the Departament de Territori i Sostenibilitat (Generalitat of Catalonia) in compliance with the National and European regulations. The PRBB animal facility has the AAALAC International approval B9900073. All the members accessing the animal house must hold the international FELASA accreditation. The Project License covering the proposed work (Ref 10642, GC) pays particular attention to the 3Rs.

Zebrafish strains

All zebrafish strains were maintained alternating generations of incrosses and outcrosses with wild-type. Embryos were obtained by mating adult fish following standard methods and grown at 28.5ºC or 23ºC. % 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich) was used as an inhibitor of pigmentation from 24hpf onward. The Tg[βactin:HRAS-EGFP] line (termed Tg[CAAX:GFP] herein) expresses GFP in the plasma membrane and was used to label the cell contours (Dale and Topczewski, 2011). Tg[vglut2a:Lox:DsRed:Lox-GFP] and Tg[gad1b:Lox:DsRed:Lox:GFP] switch lines label glutamatergic and GABAergic neurons, respectively[18]. The Tg[HuC:GFP] line labels differentiated neurons[40]. The Tg[*elavl3*:jrGECO1b] transgenic line is a reporter of neuronal activity[41]. Tg[neurog1:CreP2A-nlsCerulean] transgenic line was generated using Tol2 transgenesis.

CRISPR/Cas9-based approach to target proneural genes in F_0

Four different CRISPR single RNAs guides (sgRNAs) were used to redundantly target proneural genes following[26]. The strategy was to target 4 sites of each proneural gene CDS encompassing the bHLH DNA binding domain sequence to either generate a frame shift or a premature stop codon either prior to the DNA binding sequence or within the DNA binding sequence, or alternatively a complete excision of the DNA binding sequence (Extended Data Figure 3b). As controls, we used sgRNAs not targeting the zebrafish genome designed by randomization of antibiotic resistance gene sequences. All CRISPR RNAs (crRNAs) were ordered from Integrated DNA Technologies (IDT) (Table S1). One-cell stage embryos were injected with a Cas9/gRNAs mix, containing IDT Alt-R HiFi S.p. Cas9 Nuclease V3 (62μM) and a 4 gRNAs mix made from IDT

crRNA and tracrRNA annealing (100μM). Embryos were injected with ~1nl of 9μM RNP with a 1:1 Ratio of Cas9 and gRNA incubated at 37°C for 5min and chilled on ice before injection[26]. Deletion efficiency was assessed by gDNA PCR amplification of injected embryos. Disruption of the CDS was considered complete when no band of the original sequence length was observed, and partial when a faint band of the original sequence length was still visible. Combinations of gRNAs were tested until they led to most of the complete disruption in 10/10 samples.

Whole mount Hybridization Chain Reaction

Zebrafish embryos at selected stages were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, dehydrated through a methanol series (25%, 50%, 75%, 100%), and stored at −20 °C until use. After rehydration, embryos were permeabilized with proteinase K (10 µg/ml in PBST; PBS with 0.1% Tween-20) for 10 min at 24 hpf or 20 min at 48 hpf, followed by post-fixation in 4% PFA for 20 min at room temperature. HCR™ Gold RNA-FISH was performed according to the manufacturer's instructions (Molecular Instruments, Los Angeles, CA). Custom probe sets targeting neurog1 (X1), ptf1a (X2), atoh1a (X7), Cre (X7), and asc1b (X3) were used with corresponding amplifiers (X1–488, X2–546, X7–514, X3–647). Hybridization was carried out overnight, followed by overnight incubation with amplifiers. Embryos were imaged within 3 days using a SP8 Leica inverted confocal microscope.

Confocal imaging of whole mount embryos

Zebrafish embryos were treated with 1% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich) as an inhibitor of pigmentation from 24hpf onwards. Then, they were anesthetized with tricaine and mounted dorsally in 1% low melting point agarose with the hindbrain positioned towards the glass-bottom Petri dish (Mattek) at the desired time. Images were acquired with either a Leica SP8 or Stellaris8 system using 20×glycerol immersion objective, HCX PL APO Lambda blue 20×/0.7 argon laser 30%-80%, DPSS 561; Ready Lasers. z-stacks were recorded with 0.57×0.57 × 1.04 µm voxel size. The gain was adjusted to each signal to cover the full range of pixel values, minimize signal to noise ratio and compensate inter-individual variability.

Confocal image processing

Briefly, confocal images were processed using a standardized workflow in which they were 1) deconvoluted using a Richardson-Lucy algorithm and a theoretical PSF, 2) cropped and flipped

to be in a consistent orientation and to reduce weight of duplicated images, 3) aligned to a common reference using our rigid alignment software (RAD), 4) segmented at the single cell level using Cellpose[42], 5) masks were intersected to identify cells that strictly expressed Cre prior to the neurotransmitter and cell count was obtained, and 6) masks of 10 embryos were averaged to generate a population spatial distribution 3D model.

Light Sheet Fluorescence Microscopy

For developmental recordings Tg[neurog1:Cre; vglut2/gad1b:switch] embryos anesthetized with tricaine were mounted in 0.35% LMP-agarose within a V-shape chamber filled with fish water. Imaging was performed every 5 min for 24h at 28.5°C on a Viventis microscope using 16x objective. 445nm, 488nm and 560nm channels were simultaneously recorded for 3 embryos every 5 min.

For calcium imaging, Tg[elavl3:jrGeco1b] embryos were screened for the fluorescent transgenic indicators, immobilized by immersion in E3 water containing alpha-bungarotoxin (1mg/mL) for 2min and rinsed in E3 water for 20min until unresponsive to touch[43,44]. Embryos were then embedded in 2% low melting point agarose (Type VII, Sigma-Aldrich) prepared in filtered fish water and encased within a custom-designed glass capillary (2 mm outer diameter, 20 mm length; Hilgenberg GmbH). The agarose encased embryo was then partially extruded out of the capillary to expose the embryo head. The capillary itself was mounted vertically in the imaging specimen chamber of a SiMView light-sheet microscope filled with filtered fish water maintained at 28.5°C, with the dorsal side of the embryo facing the detection objective (Nikon 16x/0.8 NA). A volume of ~800×400×200µm^3 (X×Y×Z) was acquired with a Z spacing of 2.2µm at a rate of 2Hz, using a 5ms exposure time per image and an illumination laser wavelength of 561nm with 4mW beam power and 2.5µm beam diameter at the sample location. Embryos were recorded for 30min.

Calcium Imaging analysis

Each data set was processed with a MATLAB pipeline previously described[45], and then further processed and integrated with our other datasets as follows. Briefly, recordings were first converted from the binary file format enabling such high-speed data acquisition to conventional .tiff files, a rolling window defined reference of intensity at regular intervals was used for further deltaF/F computations, and finally deltaF/F data sets were filtered using a

median filter of kernel 3 to remove noise. Using a Fiji macro, they were cropped to save storage space and simplify downstream processing, as well as re-scaled to standardize their magnification with respect to our confocal datasets. For the resulting recordings, the first frame was then used to perform the alignment in our software and the transformation matrix was then automatically applied to each frame of the recording. We then generated a projection over the temporal dimension resulting in a 3D stack representing the most intensely active neurons. This stack was then used to perform a single cell segmentation using Cellpose. Then, individual cell masks were used to trace the fluctuations in intensity across time at those coordinates.

Rigid Alignment with Deep Learning (RAD)

To align images from multiple samples and modalities we developed a Python-based interface with integrated neural network and manual 3D rigid registration capabilities. In brief, it leverages the SITK Python library to apply the computed transformation component generated either through a Pytorch based neural network or through manual user input. It offers different possibilities for neural network training, either leveraging manual inputs or a folder of original and pre-aligned images. It is designed to handle the 3D rigid registration of samples batches as well as the drift correction and jitter correction of 3D timeseries. A set of previously aligned images[7] was used to prepare a training dataset containing about 40 combinations of reference and aligned images. The training dataset was further amplified by 5-fold using random transformations (within the range of traditional alignment) allowing to largely extend the range of transformations visible to the neural network during training. The trained model was then used to process each alignment, and each registration was observed and manually adjusted within the UI if deemed necessary before being saved. When performing alignment across modality, a first sample was often aligned manually and then used as reference for other samples of that modality to spare the training of a cross-modality model.

Statistics and reproducibility

Cell counts were obtained using single cell segmentation mask from Cellpose. The trained Cellpose model and script used to generate the training dataset are available upon request.

Statistical analysis was performed in GraphPad Prism 9. For each quantified experiment, the number of replicates/embryos (n) and P value is indicated in the figure legends, if applicable. The experiments reported here were repeated independently at least three times to generate the analyzed dataset. Data distribution was not assumed to be normal; a homoscedasticity test was performed prior to the statistical test selection. A z-score mediated outlier detection across all measured cell counts was used to rule out samples and homogenize sample set to 10 from an original pool containing from 15 to 13 embryos for each condition and transgenic background. Data analyses were performed using automated procedures without consideration of experimental groups. Data analysis was performed using the same parameters across groups.

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COMPETING INTEREST'S STATEMENT

The authors declare no conflict of interests.

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DATA AVAILABILITY

All relevant data can be found within the article and its supplementary information. For more information or any specific tool, you can contact us. All code is freely available in https://github.com/cristinapujades/Blanc_et_al_2025.

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FIGURES

Figure 1

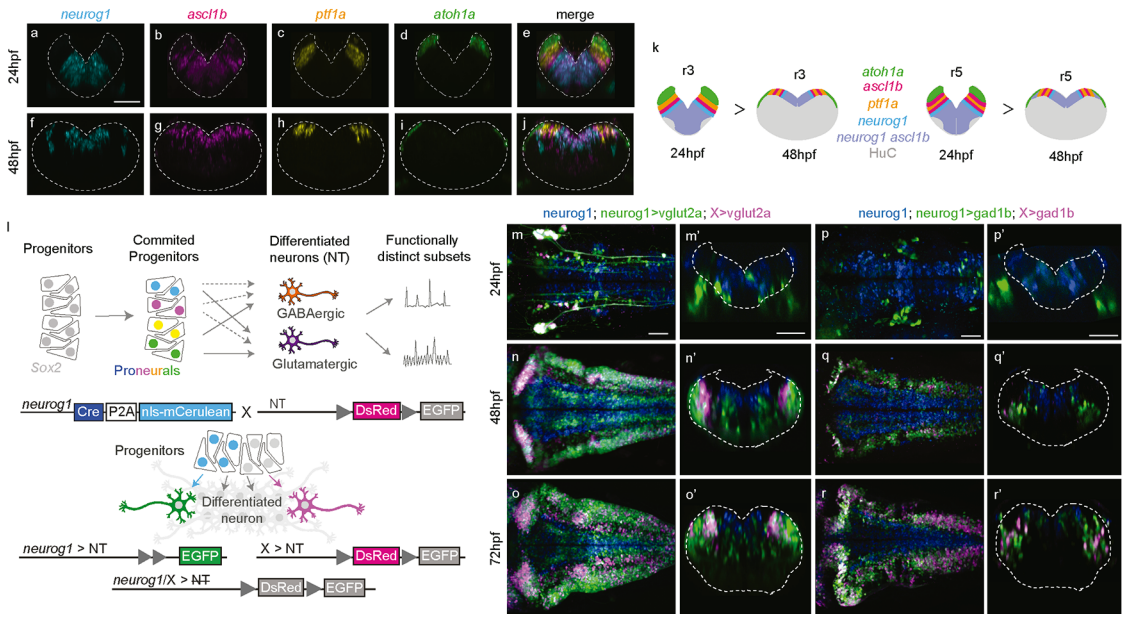


Figure 1: *neurog1*-progenitors differentially contribute to glutamatergic and GABAergic lineages.

a–j, Multiplex HCR of *neurog1, asclc1b, ptf1a* and *atoh1a* proneural genes at 24hpf (**a–e**) and 48hpf (**f–j**). Transverse views of single channels (**a–d, f–i**), and the merge of all channels (**e**, **j**). Note the differential expression of proneural genes along the dorsoventral (DV) axis at 24hpf and mediolateral (ML) axis at 48hpf. Transverse views through rhombomere (r) 5. **k,** Scheme summarizing the differential expression of proneural genes along the DV and ML axes of the hindbrain together with the neuronal differentiation domain (HuC) along rhombomeres 3 and 5. **l,** Scheme depicting the goal of the work, dissecting the contribution of different progenitor pools to excitatory (glutamatergic) and inhibitory (GABAergic) functional neurons, and the employed strategy to address the contribution of *neurog1*-progenitors to both neuronal lineages. **m–r, m'–r',** Temporal production of glutamatergic (**m–o**) and GABAergic (**p–r**) neurons according to the progenitor origin (*neurog1*-derivatives in green, non-*neurog1*-derivatives in magenta) at the indicated developmental times. Dorsal views with anterior to the left (**m–r**) and transverse views (**m'–r'**) through rhombomere 5. r, rhombomere; hpf, hours post-fertilization.

Figure 2

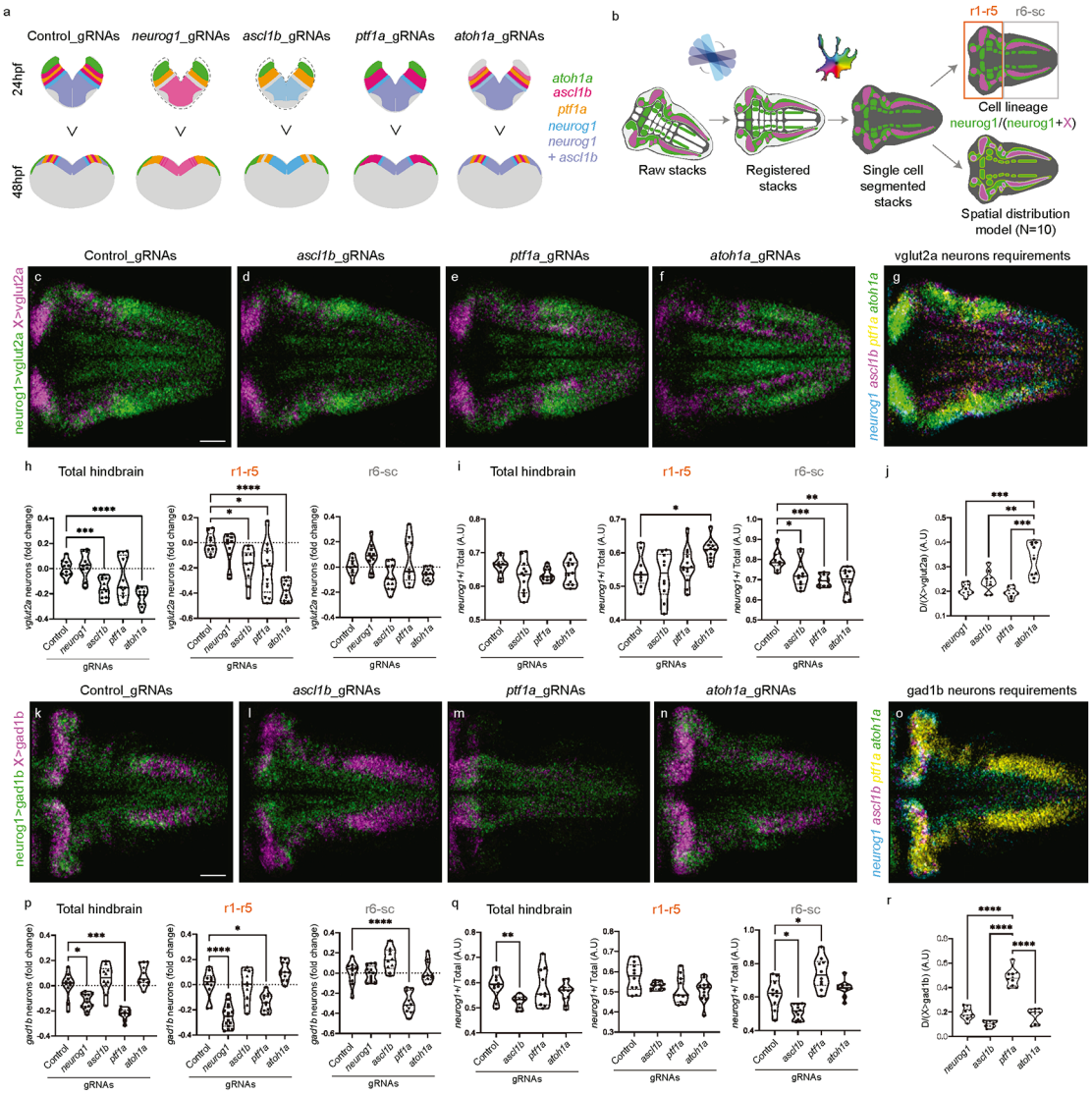


Figure 2: Glutamatergic and GABAergic lineages derive from distinct progenitor pools combinations.

a, Summary of the observed phenotypes by multiplex HCR upon proneural gene disruption (Extended Figure Data 3). **b,** Upon CRISPR-based LOF, hindbrains were imaged at 72hpf and images from 10 hindbrains per condition underwent alignment. Green (*neurog1*-derived) and magenta (non *neurog1*-derived) cells were segmented using Cellpose to *i)* quantify the proportions of glutamatergic and GABAergic neurons derived from the different progenitor populations and *ii)* construct spatial distribution 3D models allowing the evaluation of the impact of LOF on the neuronal populations across the tissue. **c–f,** Spatial distribution 3D-models of glutamatergic cells derived from *neurog1*- (green) or non-*neurog1* progenitors (magenta) upon the indicated conditions. **g,** 3D-map showing the progenitors' origin requirements for glutamatergic neurons. Note that the main contributors are *neurog1*-, *ascl1b*- and *atoh1a*-cells (see segregated progenitor Extended Data Figure 4i). **h–i,** Variation on the proportion of all glutamatergic neurons or of *neurog1*-derived glutamatergic neurons upon PN_gRNA injections with respect to the control, in the overall hindbrain (total

hindbrain), and specifically in the anterior hindbrain (r1-5) and the posterior hindbrain (r6-sc). **j,** Proportion of the non-*neurog1* derived glutamatergic neurons requirement for each of the proneural progenitor pools, measured through the intersection of control samples with the lineage requirements maps. **k–n,** Spatial distribution 3D-models of GABAergic cells derived from *neurog1*- (green) or non-*neurog1* progenitors (magenta) upon the indicated conditions. **o,** 3D-map showing the progenitors' origin requirements for GABAergic neurons. Note that the main contributors are *neurog1*-, *ptf1a*-cells (see segregated progenitor maps in Extended Data Figure 4j). **p–q,** Variation on the proportion of all GABAergic neurons or of *neurog1*-derived GABAergic neurons upon PN_gRNA injections in respect to the control, in the overall hindbrain (total hindbrain), and specifically in the anterior hindbrain (r1-5) and the posterior hindbrain (r6-sc). **r,** Proportion of the non-*neurog1* derived GABAergic neurons requirement for each of the proneurals' progenitor populations, measured through the intersection of WT samples with the lineage requirements maps. All images display dorsal views of the hindbrain with anterior to the left. r, rhombomere.

Figure 3

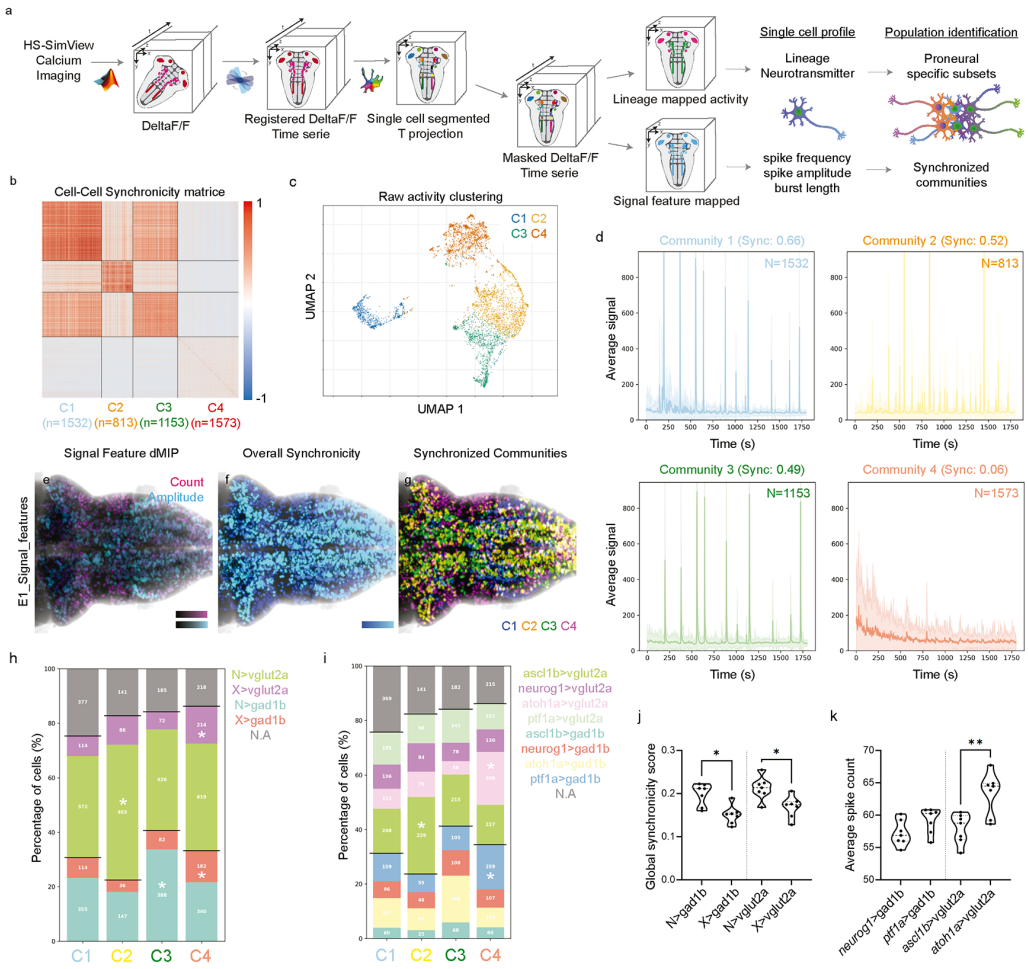


Figure 3: Functional neuronal features rely on the progenitor origin.

a, Scheme depicting the imaging and analyses pipeline: hs-SimView recordings are first processed through a series of Matlab scripts to extract the deltaF/F, and the subsequent recording is then aligned and scaled to our reference atlas using our alignment software. Aligned recordings of deltaF/F are then projected over time to obtain a 3D image representing neuron maximal activity over the time sequence. The projection is passed to Cellpose for single cell segmentation and intersected with our previously established lineage maps. Moreover, each cell's deltaF/F signal variations over the time sequence are analyzed to obtain feature maps reflecting the cells' activity characteristics. **b,** Cell-cell synchronicity matrices of hindbrain active neurons; n, number of segmented cells constituting each community. Note that we can differentiate 4 different neuronal communities (C1–C4). **c,** UMAP displaying the 4 different communities according to their synchronicity pattern. **d,** Neuronal activity profiles of C1–C4 indicating the number of analyzed cells (N) and the synchronicity score (Sync). **e–g,** 3D-maps of active neurons according to different features: count and amplitude (**e**), overall synchronicity (**f**), community belonging (**g**). **h,** Box plot displaying the cell lineage composition of C1–C4. **i,** Box plot displaying the progenitor origin requirements of C1–C4. **j,** Dot plot showing that the global synchronicity score relies on progenitor origin. **k,** Dot plot showing that the average spike count is independent of the progenitor origin.

Figure 4

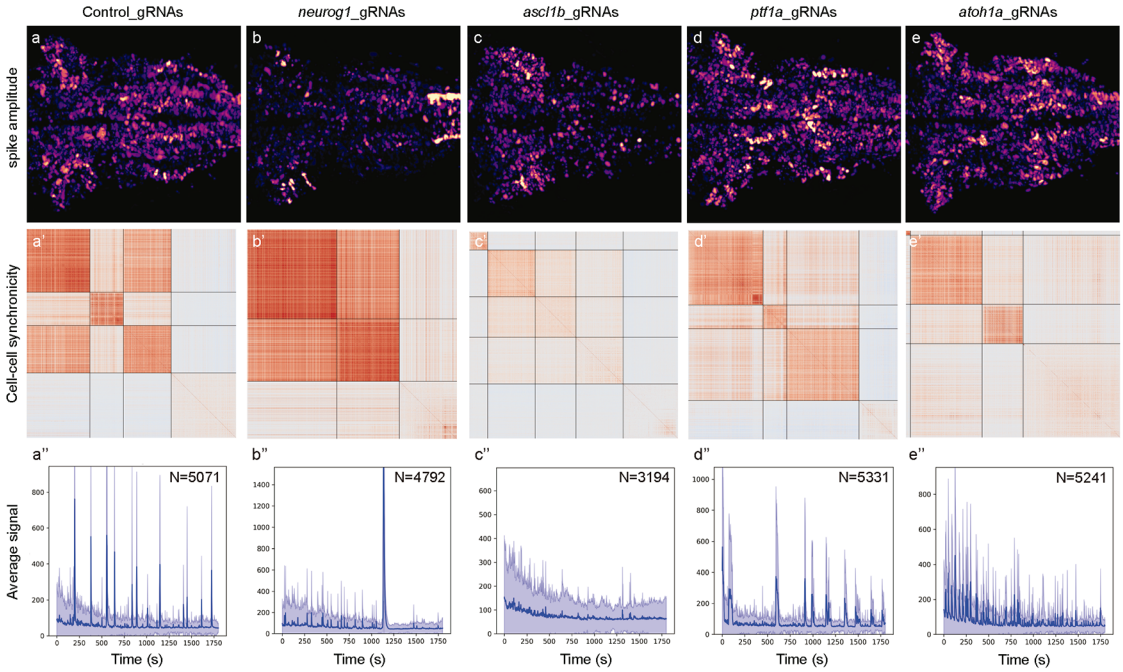


Figure 4: Disruption of specific progenitor pools results in differential changes in neuronal activity.

a–e, Neuronal activity patterns in control hindbrains and upon disruption of proneuronal progenitor pools (Extended Data Video 6–10). Dorsal views with anterior to the left. **a'–d',** Cell-cell synchronicity maps showing the 4 Louvain communities upon different conditions. **a''–d'',** Average hindbrain neuronal activity under the different conditions. Note how disruptions in the progenitor origin result in changes in community distribution and neuronal activity.

Figure 5

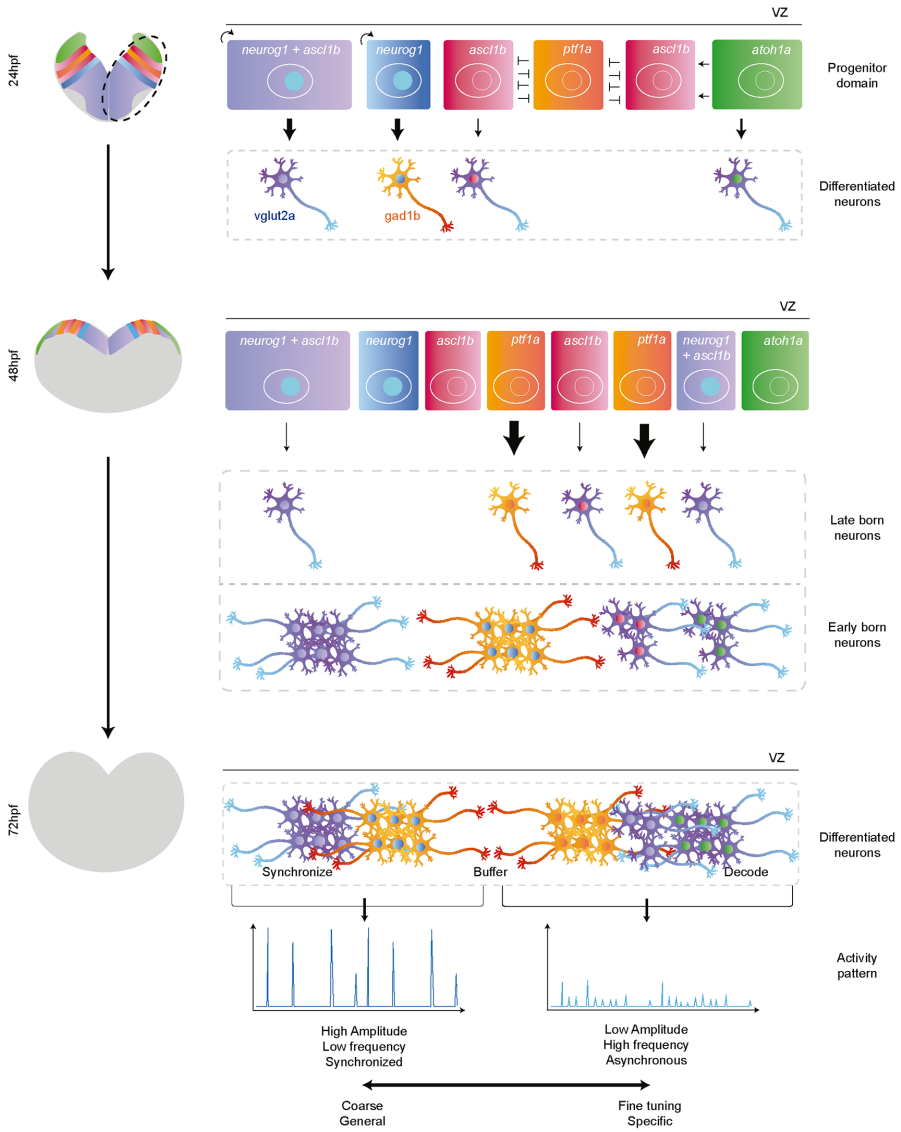
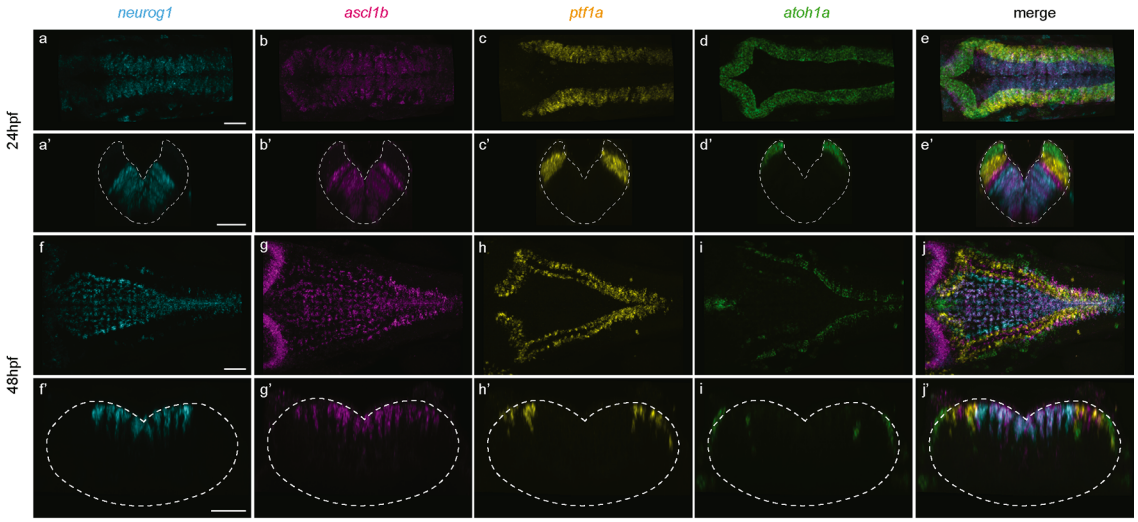


Figure 5: Progenitor origin and age are crucial parameters in establishing terminal characteristics, such as the neurotransmitter identity and function.

The neuronal progenitor domain is patterned along the DV and ML axes by differential proneural gene expression. Early on (24hpf), *neurog1*-stem cells are the first one to produce excitatory (glutamatergic, vglut2a) and inhibitory (GABAergic, gad1b) neurons with differential temporal dynamics, followed by *atoh1a*- and *ascl1b*-progenitors, which are continuously added to the glutamatergic population. On the other hand, *ptf1a*-stem cells take the relay for GABAergic neurons (48hpf). This temporally defined stem cell origin determines specific functional neuronal features: early-born, medially located stem cells produce neurons characterized by high-amplitude, low-frequency, and synchronized activity, likely establishing the baseline circuit architecture; by contrast, later-born, laterally located stem cells produce neurons with low-amplitude, high-frequency, and asynchronous activity, suggesting a role in more specific fine-tuning. VZ, ventricular zone; hpf, hours post-fertilization.

EXTENDED DATA FIGURES

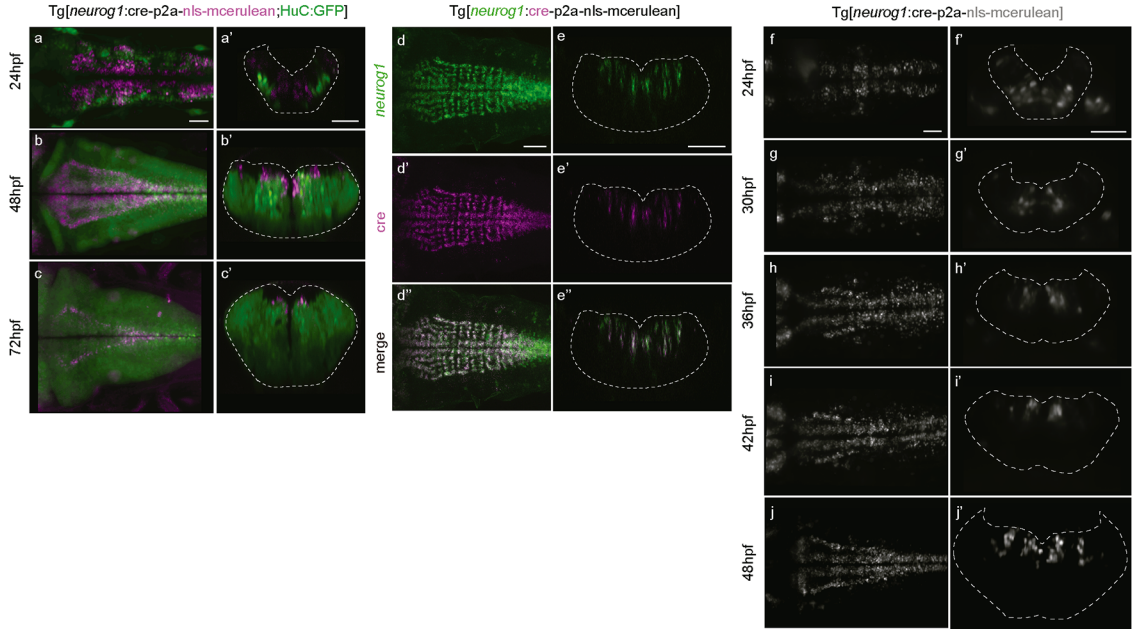
Extended Data Figure 1



Extended Data Figure 1: Proneural genes are differentially expressed along the DV and ML axis within the hindbrain.

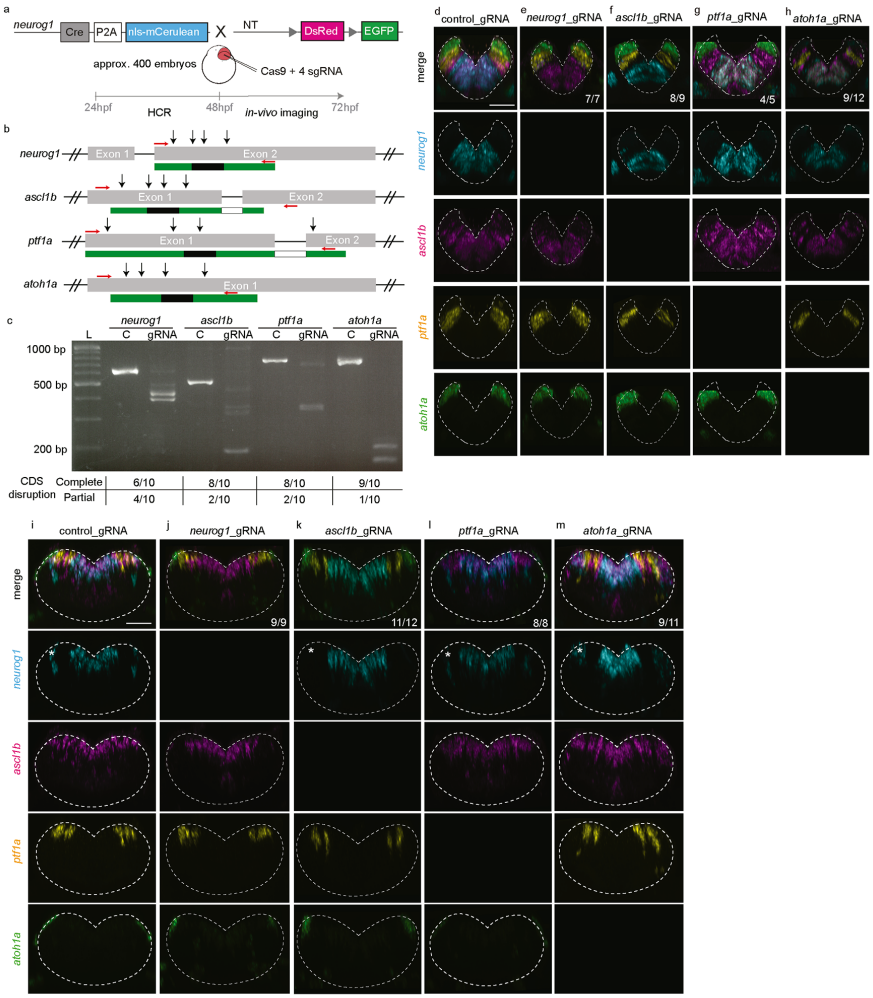
a–j, a'–j', Multiplex HCR of *neurog1, ascl1b*, *ptf1a*, *atoh1a* proneural genes displaying single (**a–d, f–i**) and merged (**e, j**) channels at 24hpf (**a–e, a'–e'**) and 48hpf (**f–j, f'–j'**), in dorsal (**a–j**) and transverse (**a'–j'**) views along r3. Note that proneural genes are expressed in the progenitor domain and their expression is displaced upon the growth of the below neuronal differentiation domain. r, rhombomere; hpf, hours post-fertilization.

Extended Data Figure 2



Extended Data Figure 2: Characterization of the Tg[neurog1:Cre-P2A-nls-Cerulean] fish line. a–c, a'–c', Double transgenic Tg[neurog1:Cre-P2A-nls-Cerulean; HuC:GFP] embryos at the indicated developmental times. Dorsal maximal intensity projection (MIP) (**a–c**) and transverse section through rhombomere 5 (**a'–c'**). Note that neurog1-Cerulean expression in magenta is restricted to the progenitor domain. **d–d'', e–e'',** HCR for *neurog1* and *Cre* in Tg[neurog1:Cre-P2A-nls-Cerulean] embryos at 40hpf. Note that *Cre*-positive cells overlap with *neurog1*-cells. **f–j, f'–j',** Expression of Cerulean protein recapitulates the dynamics of *neurog1* expression[19]. Note the stability of Cerulean protein, which is maintained in neurons deriving from *neurog1*-cells (see red asterisk). **a–c, d–d'', f–j**, Dorsal views with anterior to the left. **a'–c', e–e'', f'–j'**, Transverse views through rhombomere 5. hpf, hours post-fertilization.

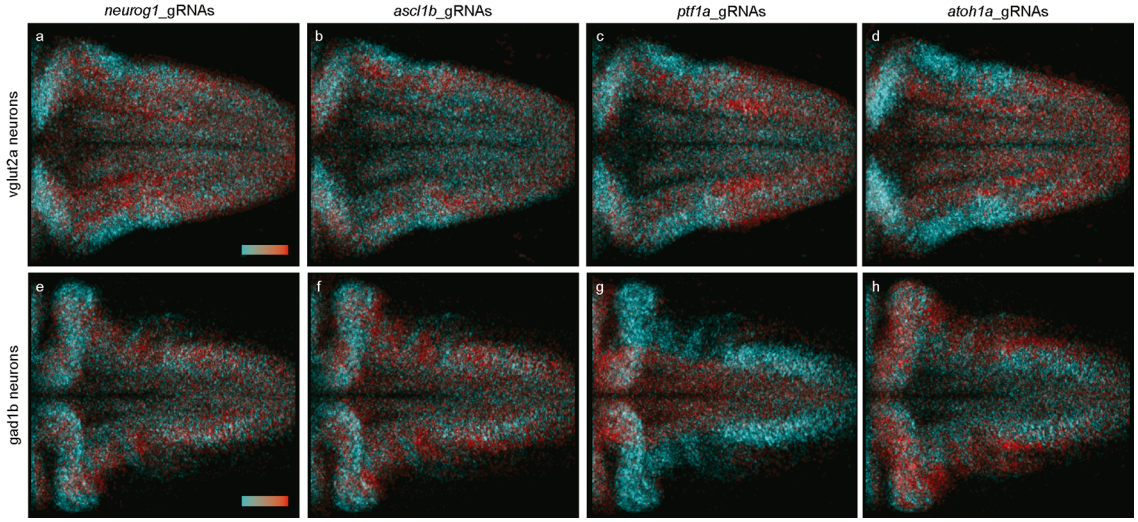
Extended Data Figure 3



Extended Data Figure 3: CRISPR-based loss-of-function approach.

a, Scheme depicting the CRISPR/Cas9-based approach that produces loss-of-function (LOF) phenotypes in F_0. Tg[neurog1:Cre:*vglut2a*/*gad1b*:switch] embryos were injected with control or 4 different gRNAs for a single proneural gene (PN_gRNAs) and they were let to develop until 24hpf and 48hpf for HCR proneural gene expression analysis, and 72hpf for cell lineage studies (Figure 2). **b,** Strategy for the proneural gene gRNAs design. Black arrows indicate the position of the 4 gRNAs within the corresponding proneural gene. Red arrows indicate the position of the PCR primers used for genotyping. **c,** Genotyping of PN_gRNAs injected embryos. gDNA from single embryos injected with control (C) of PN_gRNAs (gRNA) were used for genotyping (see Methods). Agarose gel displaying the amplified bands. The number of embryos displaying complete or partial disruption of the amplified coding sequence (CDS) after PN-gRNAs injection is indicated in the table. Efficiency of the gene disruption was assessed in single embryos at F_0. **d–h, i–m**, Multiplex HCR of *neurog1, asclc1b, ptf1a* and *atoh1a* proneural genes at 24hpf and 48hpf, respectively, upon control (**d, i**) or PN-gRNA injection (**e–h, j–m**). Transverse views through rhombomere 5 displaying the indicated single channel or the merge of them. White asterisk in the *neurog1*- profiled embryos (**i–m**) indicate the loss of expression of *neurog1*- in the most lateral domain of expression upon *ascl1b* loss-of-function.

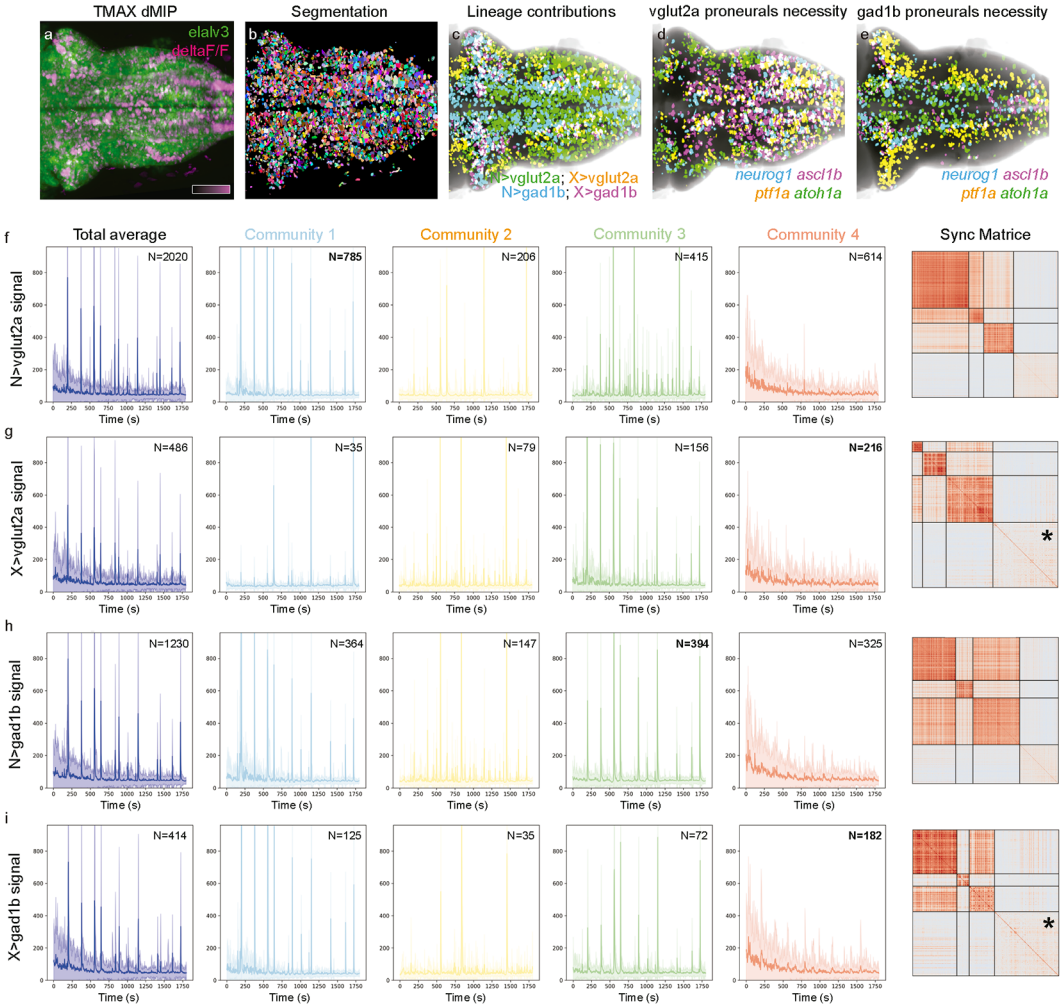
Extended Data Figure 4



Extended Data Figure 4: Differential requirement of progenitor pools for glutamatergic and GABAergic lineages.

a–f, 3D-maps displaying the progenitor origin requirement (cyan) and compensation (red) for glutamatergic (**a–d**) and GABAergic (**e–h**) neurons (see hue legend). Note that the main progenitor's requirements for glutamatergic neurons are *ascl1b*- (medial) and *atoh1a*- (lateral), whereas to the GABAergic neurons are *ptf1a*-progenitors. All images display dorsal MIP with anterior to the left. r, rhombomere.

Extended Data Figure 5

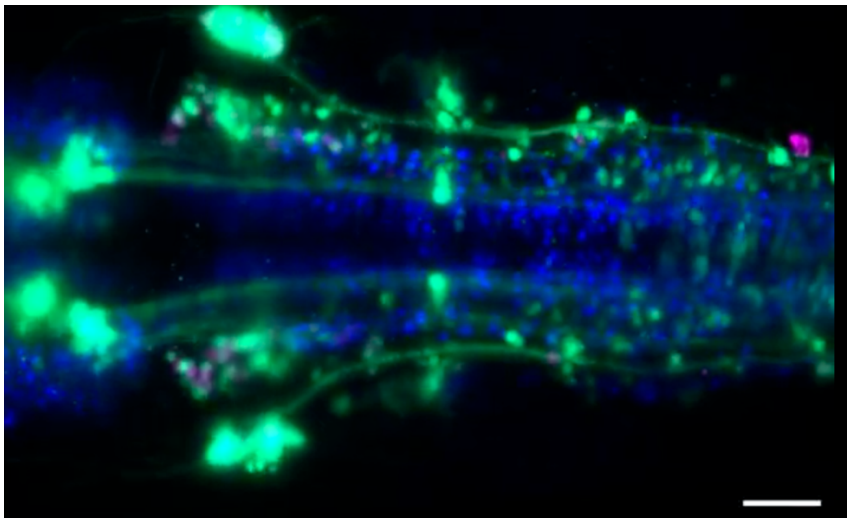


Extended Data Figure 5: Mapping lineage origin and functional activity features to a common hindbrain 3D reference map.

a, Dorsal view of Tg[*elavl3*:jrGECO1b] embryo upon Ca^{++}-recording within a 72hpf hindbrain (Extended Data Video 6). **b,** Dorsal view displaying all active segmented neurons. **c,** 3D-map displaying the contribution of *neurog1*-progenitors (green and cyan cells) and non *neurog1*-progenitors (yellow and magenta cells) to glutamatergic (green and yellow) and GABAergic (cyan and magenta) lineages. **d–e,** 3D-maps of the proneural gene requirements for glutamatergic (**d**) and GABAergic (**e**) lineages. **f–i**, Neuronal activity profiles of all glutamatergic (**f–g**) and GABAergic (**h–i**) cells (total average) and segregated by community belonging (C1–C4) from *neurog1*-progenitors (**f, h**) or non *neurog1*- progenitors (**g, i**) with the corresponding cell synchronicity matrix. **j–m,** 3D-maps of active neurons belonging to the different communities (C1–C4).

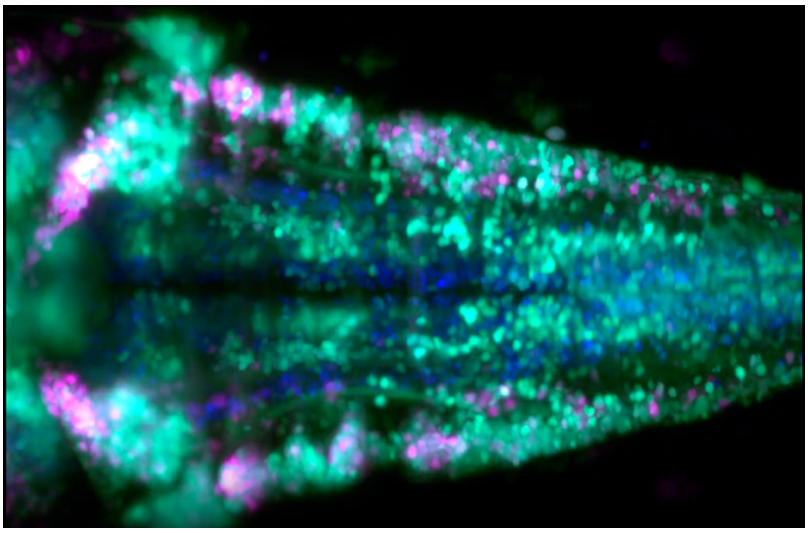
EXTENDED DATA VIDEOS

Extended Data Video 1



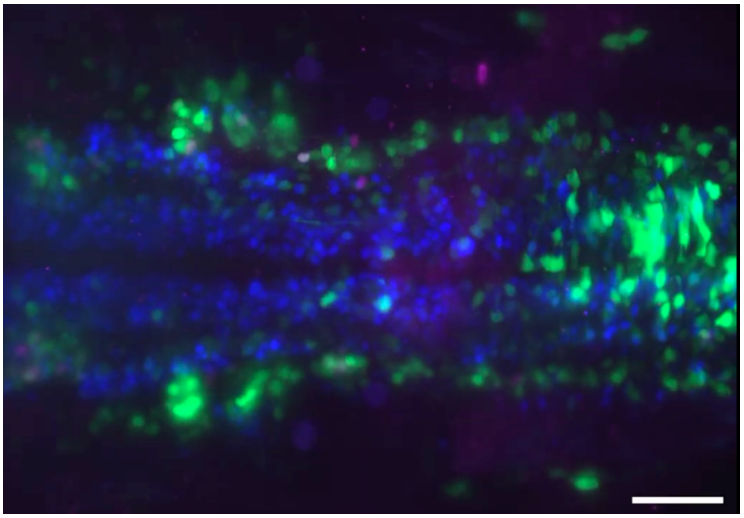
Extended Data Video 1: *neurog1*-population produces glutamatergic neurons at very early stages.
Tg[neurog1:Cre:*vglut*:switch] embryo was imaged from 24hpf until 48hpf using LSFM. Note that *neurog1*-progenitors express Cerulean and when they differentiate into glutamatergic neurons, they start expressing GFP (green), whereas the rest of glutamatergic neurons are magenta.

Extended Data Video 2



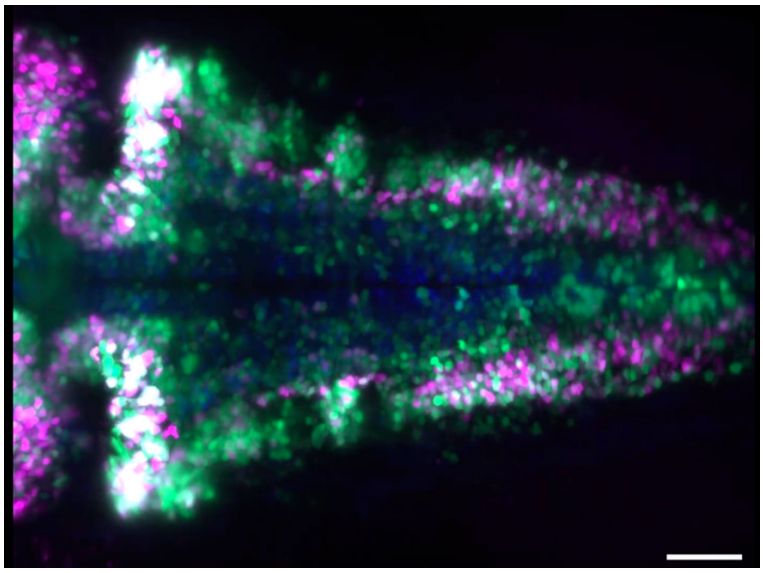
Extended Data Video 2: *neurog1*-population and other progenitor pools contribute to glutamatergic neurons at later stages.
Tg[neurog1:Cre:*vglut*:switch] embryo was imaged from 48hpf until 72hpf using LSFM. Note that *neurog1*-progenitors express Cerulean and when they differentiate into glutamatergic neurons, they start expressing GFP (green), whereas the rest of glutamatergic neurons are magenta.

Extended Data Video 3



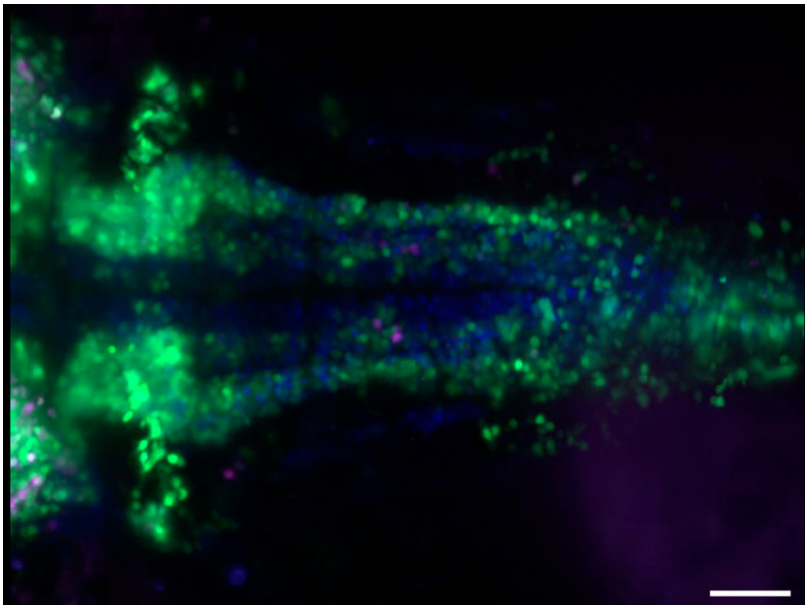
Extended Data Video 3: *neurog1*-population produces GABAergic neurons at early stages
Tg[neurog1:Cre:*gad1b*:switch] embryo was imaged from 24hpf until 48hpf using LSFM. Note that *neurog1*-progenitors express Cerulean and when they differentiate into GABAergic neurons, they start expressing GFP (green), whereas the rest of GABAergic neurons are magenta.

Extended Data Video 4



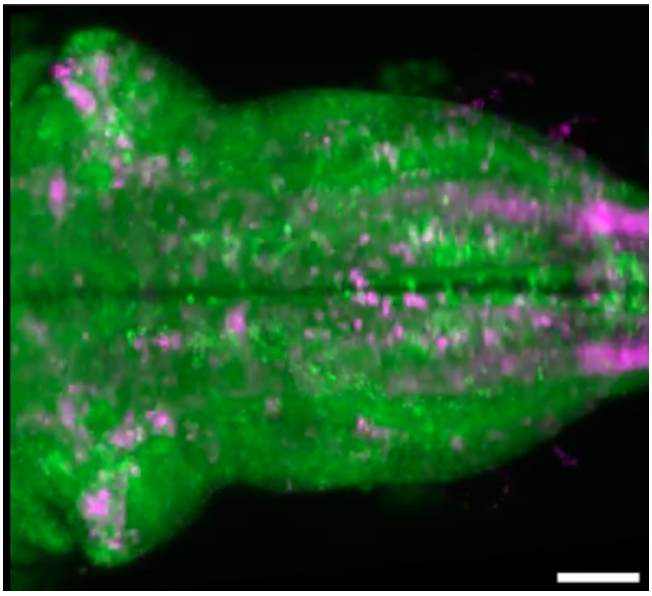
Extended Data Video 4: *neurog1*-population produces GABAergic neurons at early stages
Tg[neurog1:Cre:*gad1b*:switch] embryo was imaged from 48hpf until 72hpf using LSFM. Note that non *neurog1*-progenitors (magenta cells) take the relay of neurog1-progenitors in the production of GABAergic neurons.

Extended Data Video 5



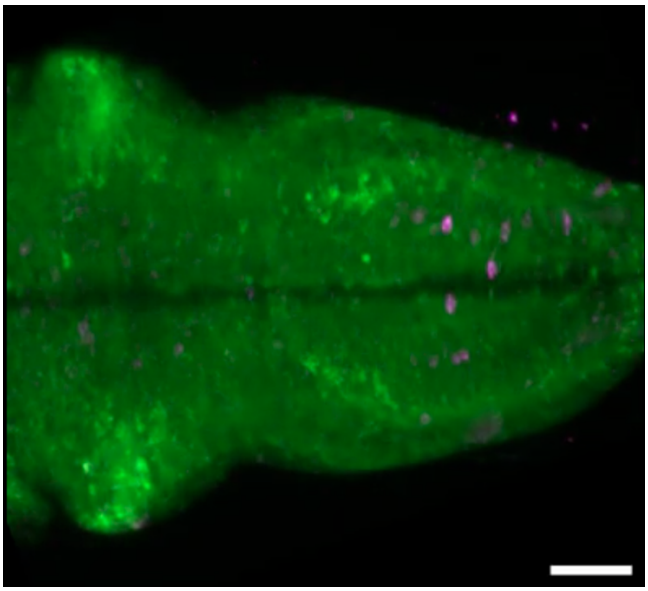
Extended Data Video 5: GABAergic neurons are generated independently of *ptf1a*.
Tg[neurog1:Cre:*gad1b*:switch] embryo injected with 4 different gRNAs for *ptf1a* imaged from 48hpf until 72hpf using LSFM. Note that indeed *neurog1*-cells give rise to GABAergic neurons in the absence of *ptf1a.*

Extended Data Video 6



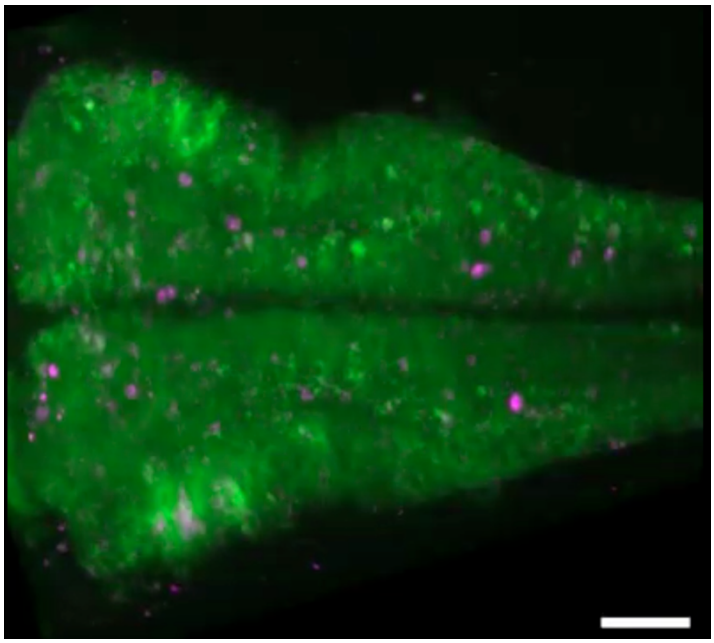
Extended Data Video 6: Neuronal activity pattern in the hindbrain in control embryos.
Calcium recordings in Tg[*elavl3*:jrGECO1b] embryos at 72hpf after control gRNA injections. Note how the Ca^{++}-activity pattern changes upon disruption of different progenitor pools. Dorsal view with anterior to the left.

Extended Data Video 7



Extended Data Video 7: Neuronal activity pattern in the hindbrain upon disruption of *neurog1*-progenitors.
Calcium recordings in Tg[*elavl3*:jrGECO1b] embryos at 72hpf after *neurog1*-gRNA injections. Note how the Ca^{++}-activity pattern changes upon disruption of *neurog1*-progenitors. Dorsal view with anterior to the left.

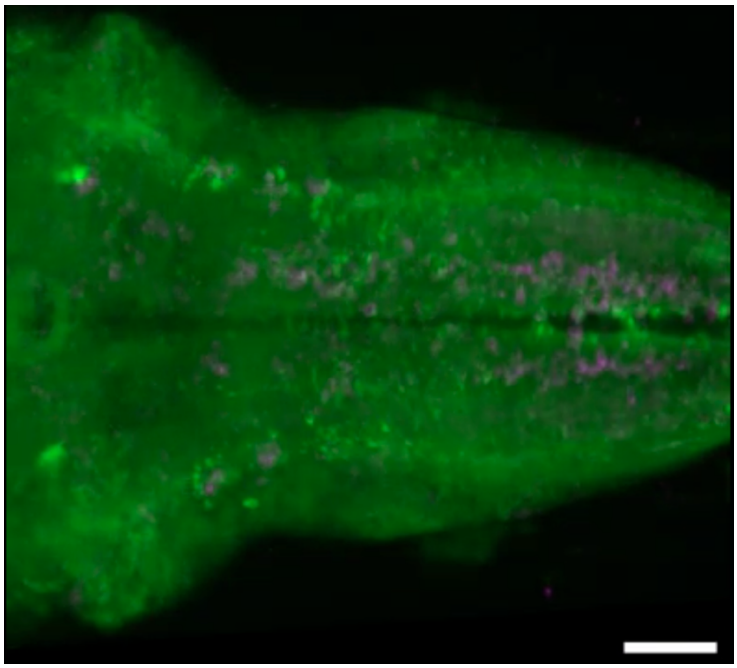
Extended Data Video 8



Extended Data Video 8: Neuronal activity pattern in the hindbrain upon disruption of *ascl1b*-progenitors.

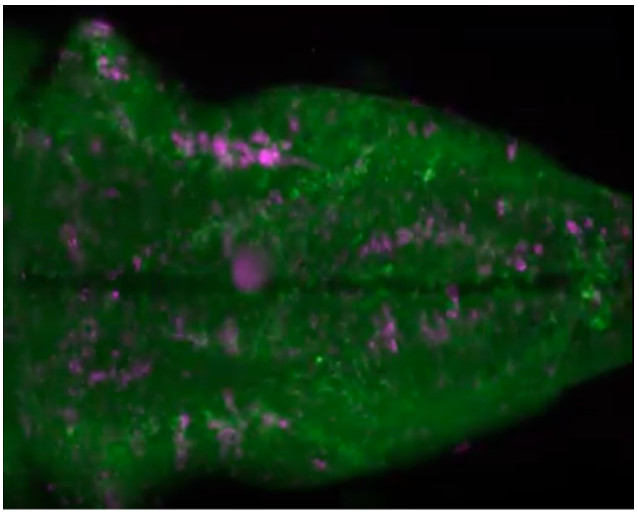
Calcium recordings in Tg[*elavl3*:jrGECO1b] embryos at 72hpf after *ascl1b*-gRNA injections. Note how the Ca^{++}-activity pattern changes upon disruption of *ascl1b*-progenitors. Dorsal view with anterior to the left.

Extended Data Video 9



Extended Data Video 9: Neuronal activity pattern in the hindbrain upon disruption of *ptf1a*-progenitors.
Calcium recordings in Tg[*elavl3*:jrGECO1b] embryos at 72hpf after *ptf1a*-gRNA injections. Note how the Ca^{++}-activity pattern changes upon disruption of *ptf1a*-progenitors. Dorsal view with anterior to the left.

Extended Data Video 10



Extended Data Video 10: Neuronal activity pattern in the hindbrain upon disruption of *atoh1a*-progenitors.
Calcium recordings in Tg[*elavl3*:jrGECO1b] embryos at 72hpf after *atoh1a*-gRNA injections. Note how the Ca^{++}-activity pattern changes upon disruption of *atoh1a*-progenitors. Dorsal view with anterior to the left.