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The GABAergic and Mixed GABAergic/glycinergic Neurons of the Murine Cerebellar Nuclei: What the Genes Tell Us

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Our knowledge of the cellular composition and internal wiring of the cerebellar nuclei lags considerably behind our understanding of the cerebellar cortex. Here, we take advantage of publicly available data of gene expression in cells of the cerebellar nuclei and cortex to compare and contrast GABAergic and mixed GABAergic / glycinergic cells of these territories. We find that nucleo-olivary projection neurons are characterized by dichotomic expression levels of multiple genes, but in contrast to zebrin II-defined Purkinje cells, may not be separated into clearly distinct sets. The gene expression patterns of previously identified sets of inhibitory nuclear interneurons resemble those of Golgi, globular and Lugaro cells. Our analysis also suggests that the distinctive electrophysiological characteristic of inhibitory "interneurons" which also target the cerebellar cortex may be due to their quite specific expression of glycine receptors.

Key words: cerebellum, cerebellar nuclei, inhibitory interneuron, gene expression, scRNA

Introduction

The cerebellum is often used as a textbook paradigm to introduce students to basic principles of the structure, function, development and pathology of the central nervous system. Its cortex may be described as an extremely regular, quasi-crystalline, three-

layered ensemble of only a few cell types, with readily distinguishable morphologies, which are wired in a highly stereotyped manner. Purkinje cells arguably form the pivot of the cerebellar cortical circuitry. They directly receive extra-cerebellar input through climbing fibers, and indirectly through mossy fibers. The latter is mediated and modulated by granule cells. The cerebellar cortical circuitry is complemented by a set of inhibitory (i.e., GABAergic and/or glycinergic) interneurons, viz. Golgi cells in the granule cell layer, Lugaro cells, globular cells and candelabrum cells resident in the upper granule cell and Purkinje cell layers, and basket and stellate neurons in the molecular layer. Thus, cerebellar cortical inhibitory interneuron somata are located in quite distinct environments: whereas the molecular layer is formed primarily by a dense entanglement of granule cell axons, dendrites of inhibitory interneurons and Purkinje cells, and the processes of Bergmann glial cells, the granule cell layer and the Purkinje cell layer comprise a very dense matrix of granule cell and Purkinje cell somata. Cortical inhibitory interneurons may be further distinguished by their (primary) axonal projections to targets in the granule cell layer (i.e., Golgi cells), in the molecular layer (i.e., stellate cells, and Lugaro, candelabrum and globular cells), or in the molecular and Purkinje cell layers (basket cells; for a reviews, see [31, 33]). Lastly, cerebellar cortical inhibitory neurons may be classified by the transmitter(s), i.e., whether they are purely GABAergic (like basket, stellate, and a smaller subset of Golgi neurons), or also use glycine (most Golgi cells, Lugaro and globular cells; for an extended and detailed classification, see [35]).

A distinguishing characteristic of cerebellar cortical inhibitory interneurons is that their developmental diversification differs mechanistically from that of, say, inhibitory interneurons in other regions of the CNS (e.g., [24, 30]): they originate from a common pool of precursors characterized by the expression of the paired-box gene, Pax2. These precursors are programmed to their specific fate postmitotically by hitherto unknown, environmental signals while they migrate to their final destinations [18, 19], or even after arriving there [31].

In contrast to the cerebellar cortex, our understanding of the cerebellar nuclei is still rather fragmentary (for a recent review, see [14]). Typically, their role as the (sole) output gateway of the cerebellum is stressed. Output is realized through glutamatergic and glycinergic neurons, which target multiple areas in the CNS (see [14] for a detailed review), and through GABAergic cells projecting to the inferior olive. It is by now well established that cerebellar nuclei receive input not only from the cerebellar cortex, but also from extra-cerebellar sources. Importantly, they also comprise local interneurons. Thus, their cellular composition and structure strongly support the notion that they not only channel, but in fact process information.

Based on the data of Kebschull et al. [15], we may estimate that, in the mouse, inhibitory nuclear interneurons account for some 40% of all inhibitory nuclear neurons. Like inhibitory interneurons of the cerebellar cortex, inhibitory interneurons of the cerebellar nuclei are derived from Pax2-positive precursors. Yet in contrast to cortical inhibitory interneurons, the characterization of those of the cerebellar nuclei is still rather fragmentary, and essentially based on functional analyses [1, 39]. There is also evidence that some of the inhibitory neurons of the cerebellar nuclei may share characteristics of interneurons and projection neurons [1, 8, 39].

Here, we attempt to gain further insight into the diversity of cerebellar nuclear inhibitory (inter-) neurons by comparing them with those in the cerebellar cortex. We

take advantage of the fact that recent single cell gene expression studies of the [adult] cerebellum, notably those of Kebschull et al. [15] and Kozareva et al. [16], grant a novel and unique vantage point to do so. Combining these datasets allowed us to assess the affinities of cerebellar nuclear inhibitory (inter-) neurons with inhibitory neurons from the cerebellar cortex, but also to pinpoint some differences.

Materials and Methods

Gene expression data for murine cerebella published by Kebschull et al. [15] and Kozareva et al. [16] were obtained from the NCBI Gene Expression Omnibus (GEO; accession numbers GSE160471, sample GSM4873765, and GSE165371, file GSE165371_cb_adult_mouse.tar.gz). Of note, these data sets had already been subjected to stringent quality controls (see the original publications cited).

All further data processing was done using R (version 4.3.1 patched; R Core Team, 2023; available at https://cran.r-project.org/) and package Seurat (version 4.4; [10]) and visualized using package scCustomize (version 1.1.3; [23]).

From the adult expression data of the cerebellar cortex of Kozareva et al. [16], we extracted data for inhibitory neurons as per the annotation provided with this dataset. This resulted in a set comprising data for 66,390 cells. Random subsets of these cells comprising 9,000 cells were generated using the R function "sample". From the data published by Kebschull et al. [21] for cerebellar nuclei, we utilized only the subset classified by these authors as inhibitory (ventricular zone-derived), comprising 2363 cells. Before integrating these data (i.e., a given subset of inhibitory cortical neurons and inhibitory nuclear neurons), we trimmed them to those 25,134 features (genes) present in both data sets. Integration followed the standard procedure as recommended for the Seurat package (cf https://satijalab.org/seurat/articles/integration_introduction). Cell-type specific annotations shown are based on those given in the original publications describing these data.

Results

To compare gene expression in adult cerebellar inhibitory interneurons, we used data obtained for murine nuclear inhibitory cells [15] and subsets of the data reported by for cortical inhibitory neurons [16]. The decision to use only a subset of the latter dataset was motivated by fact that data for 2363 nuclear cells were available, and the consideration that these should not be outnumbered too extremely by cortical cells. We repeated the analyses reported below with several randomly selected subsets of the cortical inhibitory interneurons, and cortical subsets comprising neurons from male animals only, as did the nuclear sample. This did not appreciably affect the results reported (data not shown).

Cluster i1 (nucleo-olivary projection neurons)

Clustering of all inhibitory interneurons revealed that Purkinje cells and inhibitory nuclear neurons of cluster 1 (i1), which were previously tentatively identified as nucleo-

olivary projection neurons [15], clustered close together, with only a small overlap (**Fig. 1A**). As a group, Purkinje cells and nuclear cells of cluster i1 were well separated from all cortical inhibitory interneurons, i.e. interneurons from the molecular layer (in clusters Mli1_1, Mli1_2, and Mli_2), Golgi cells in clusters Golgi_1 and Golgi_2, and candelabrum cells, globular cells, and Lugaro cells [27]. They were also well separated from nuclear inhibitory neurons in clusters i2_2, i2_2, i2_3 and i3.

As expected, cells in the cluster representing Purkinje neurons strongly and selectively expressed known Purkinje cell-specific genes, like *Pcp2* (L7; [26]), *Calb1*, or *Car8*. They also (rather) selectively expressed *Ebf1, Arhgef33, Kcnab1, Itpr1, Stac, Sox5* and *Grid2ip*. Conversely, among the genes selectively expressed by cells of cluster i1, there were *Zfhx4* (cf [15]), *Drd2, Pou3f2, Syt6, Slc24a4, Fxyd6, Cntnap3* and, notably, *Dmbx1*. Yet in contrast to Purkinje cell specific genes *Pcp2, Calb1, Itpr1* or *Car8*, none of the i1-specific genes was expressed in all i1 cells. Indeed, they typically were expressed in some 20-50 % of the cells of this cluster. *Zfhx4* was expressed in almost all cells of i1 (1328/1401), and *Slc24a4* in 1144/1404 cells. However, no combination of any two of the genes specifically expressed in cluster i1 labeled more than 95% of its cells. To wit, the Purkinje cell markers mentioned *individually* were expressed in more than 99.7 of the cells in the Purkinje cell cluster (PC). Also, among the genes specific for cluster i1, we found no pair that was expressed in mutually exclusive subsets of this cluster. Rather, even for genes expressed in rather small subsets of cluster i1 (say, *Dmbx1*, expressed in 331 cells; and *Drd2*, expressed in 362 cells of a total of 1401 cells in cluster i1) co-expression was found in 83 cells, i.e. ~ 23% of the cells expressing either of the two genes.

Differential gene expression allows distinguishing spatially organized subsets of Purkinje cells during development (e.g., *Pcp2* / L7; cf. [25, 36] and allow defining compartments of functionally distinct subsets of Purkinje cells in the adult, as prominently documented for zebrin II / aldolase C, [3, 12]. Intriguingly, Purkinje cells positive or negative for zebrin II / aldolase C are rather well separated within the Purkinje cell cluster, attesting to the fact that levels of zebrin II expression signify a more fundamental difference in gene expression between these sets of Purkinje cells (**Fig. 1B).**

Compartmentation has also been reported for cerebellar nuclei [5, 37]. Of the (anti-) genes used to define nuclear compartments by Chung et al. [5], *Slc6a5, Tbr1* and *Ebf2* are not expressed in nucleo-olivary projection neurons (i.e., neurons of cluster i1; cf the online material associated with the publication by Kebschull et al., [15] at https://github.com/justuskebschull/CNcode_final). As documented in **Fig. 1B,** none of genes associated with nuclear clusters show a pattern of distribution in nucleo-olivary projection neurons comparable to that observed in Purkinje cells based on their differential expression of zebrin II (*Aldoc*). Rather, as visible primarily for genes expressed in smaller subsets of i1-cells, their distribution follows the overall cell density distribution in cluster i1.

To further explore this issue, we compared gene expression by cells of cluster i1 either positive or negative for *Dmbx1*. The rational for this inquiry was that *Dmbx1* is the earliest known marker for at least a subset of precursors of nucleo-olivary projection neurons (cf [14]). No appreciably significant, or suggestive, differences of gene expression, other than *Dmbx1* itself, could be ascertained in cells positive or negative for this marker.

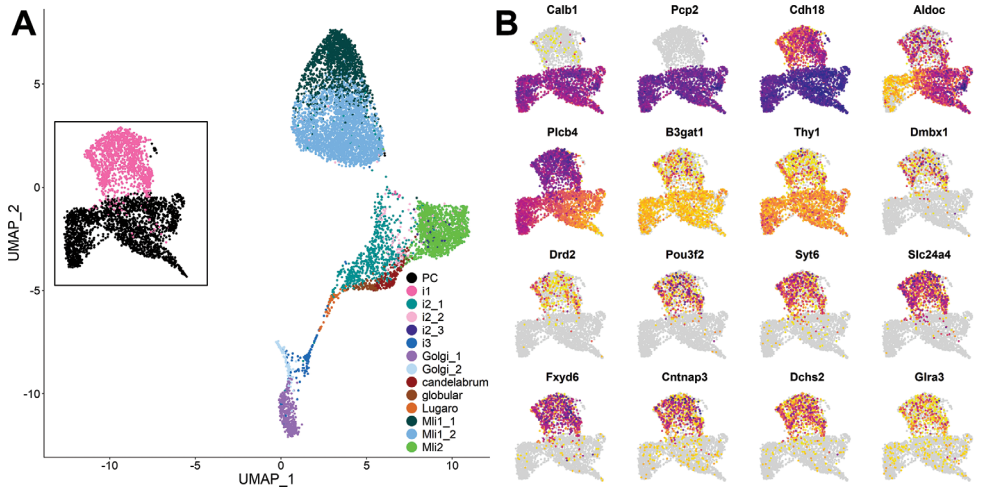

Fig. 1. Clustering of cortical and nuclear cerebellar interneurons and transcriptional heterogeneity in nucleo-olivary projection neurons. **(A)** Clustering of individual cells from adult cerebellar cortices and nuclei visualized by UMAP. Note that Purkinje cells and nucleo-olivary projection neurons (PC and i1, respectively) are well separated from nuclear and cortical inhibitory interneurons. **(B)** Examples of gene expression in clusters representing Purkinje cells and nucleo-olivary projection neurons (see boxed area in panel A) visualized by UMAP. Each dot represents an individual cell. Darker colors signify higher levels of gene expression; light gray marks negative cells. Purkinje cells can be unambiguously identified by classical markers (*Calb1, Pcp2*), and divided in two rather sharply separated sets defined by *Aldoc* (zebrin II) expression, and also by differential expression of *Plcb4*. Note also the differential expression levels in genes preferentially or exclusively expressed in nucleo-olivary neurons.

Cluster i2 and its subclusters, and cluster i3

Nuclear inhibitory neurons of clusters i2_1, i2_2, i2_3 and i3 are well separated from cells in the nuclear cluster i1 and Purkinje neurons. Strikingly, they are arrayed along a trajectory spanned out by molecular layer interneurons in cluster Mli2 and Golgi cells in clusters Golgi_1 and Golgi_2, closely following the arrangement of candelabrum cells, globular cells and Lugaro cells (**Fig. 1A**). A higher resolution view of these cells after removal of Purkinje cells, i1 cells and the molecular layer clusters Mli1_1 and Mli1_2 and re-clustering is shown in **Fig. 2**. Indeed, re-clustering of the cells of the combined datasets of cortical and nuclear inhibitory neurons typically results in clusters comprising Golgi and i3 neurons, and globular and candelabrum cells joined with i2 cells, unless the resolution parameter in the "FindClusters" function of the Seurat package was set to quite high values (not shown).

The rather small cluster i2_3 (39 cells) overlapped considerably with the molecular layer inhibitory interneurons of cluster Mli2. As may be seen in **Fig. 2**, next to cluster i2_3, clusters i2_2 and i2_3 closely abut the clusters of candelabrum and globular cells; then follows the cluster representing Lugaro cells, and, intercalated between the latter and the Golgi cell clusters, we find the nuclear cluster i3. A consistent arrangement of these clusters may be verified if data are embedded using t-distributed stochastic neighbor embedding (tSNE; not shown).

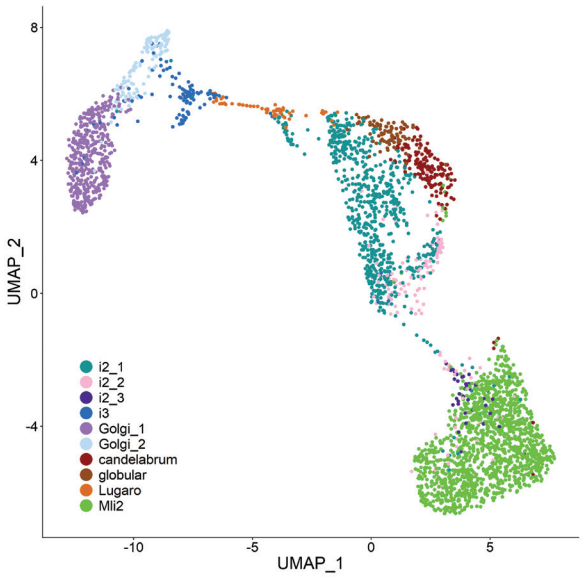

Fig. 2. Comparison of cortical and nuclear cerebellar interneurons based on their transcriptome. Cells were re-clustered after removal of projection neurons and molecular layer interneurons of clusters Mli1_1 and Mli1_2 and are visualized by UMAP. Clusters of nuclear cells are found along a line spanned out by cortical Golgi cells and molecular layer interneurons in cluster Mli2 and close to clusters of cortical candelabrum, globular, and Lugaro cells. Note also the position taken by cells of nuclear cluster i3 between Lugaro and Golgi cells.

The affinities between the cells in the individual clusters suggested by their arrangement may also be exemplified by selected markers and function-defining genes expressed by these cells as documented in **Fig. 3.** The genes shown were selected from those highly expressed in any one cluster distinguishing this cluster from all other clusters. The selection was primarily motivated by the (potential) functional significance and also includes known markers for cerebellar cells [15, 16, 22, 27, 32, 35].

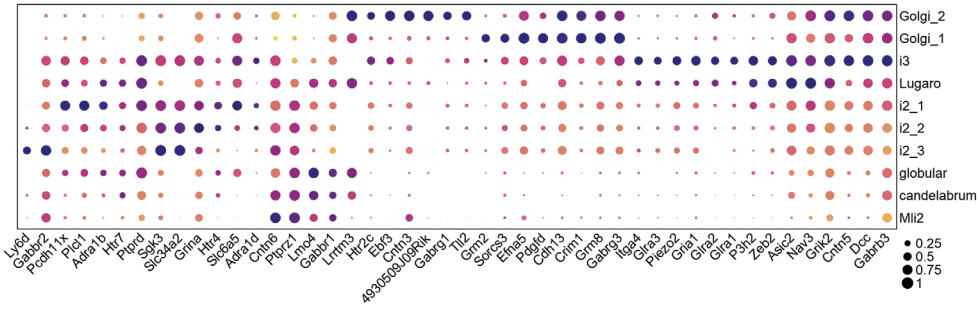

Fig. 3. Expression of selected genes documenting similarities and differences between nuclear inhibitory interneurons and cortical Golgi and Mli2 cells.While there are few genes that individually allow identifying the cell types compared (e.g., *Lyd6, Grm2*), combinations of two or three genes typically allow identification of individual cell types. Note also that in nuclear cell clusters, glycine receptor coding genes are strongly expressed rather selectively in cells of cluster i3. Among cortical cells, glycine receptor expression is more prominent in Lugaro cells. Darker colors signify higher levels of gene expression, and the symbol size indicates the fraction of cells expressing a gene (see scale at lower right corner of the figure).

As expected, there were hardly any markers that were individually specific for any one cluster *and* would label all of its cells. Yet this approach also identified small sets of genes that, when combined, allow to identify individual cell types, and to distinguish, e.g., nuclear form cortical cells. Moreover, the expression patterns of some genes also exemplify the similarities / parallels in gene expression between sets of cortical and nuclear cells as indicated, on a more encompassing scale, by the clustering patterns described above. E.g., cells of cluster i3, which are found next to the Golgi (_2) cell cluster and that of Lugaro cells **(Fig. 2)**, can be seen to share expression of *Cntn5* with the former, and *P3h2* and *Zeb2* with the latter. Yet the combined expression of either *Cntn5* and *P3h2* or *Zeb2* readily allows distinguishing i3 cells from Golgi and Lugaro cells.

A surprising and particularly intriguing result of our search for genes specific for individual cell clusters was the observation that cells of cluster i3 stand out due to their strong and broad expression of the mRNAs coding alpha subunits (primarily alpha2) and the beta subunit of glycine receptors (*Glra1, Glra2, Glra3, Glrb*). To follow up on this finding, we counted cells expressing any one of the *Glra* subunits, and the *Glrb* subunit, in all cells of our joint data set, and separately also in those cells positive for *Slc6a5*, i.e. glycinergic (inter-) neurons. The separate assessment of *Slc6a5*-positive cells was motivated by the findings of Uusisaari and Knöpfel [39], who have identified a subset of *Slc6a5*-positive cells in the cerebellar nuclei, traditionally considered to be interneurons, that also project to the cerebellar cortex. The numbers obtained are listed in **Table 1**.

Table 1. Total numbers of cells in defined clusters from the cerebellar cortex or nuclei, and of subsets positive for Slc6a5 or various glycine receptor subunits, or combinations thereof.

Cluster	Total #	*Slc6a5*+	*Glra*+	*Glra*+ and *Glrb*+	*Slc6a5*+, *Glra*+ and *Glyrb*+
GABAergic projection neurons					
PC	2342	16	345 (188)	290 (144)	1 (0)
i1	1401	42	720 (307)	555 (236)	22 (6)
nuclear GABAergic and/or glycinergic interneurons					
i2_1	675	548	235 (25)	201 (23)	166 (21)
i2_2	137	39	36 (5)	30 (3)	12 (0)
i2_3	39	1	11 (1)	7 (1)	0
i3	111	94	93 (69)	79 (59)	69 (52)
cortical GABAergic and/or glycinergic interneurons					
Golgi_1	435	379	97 (95)	84 (82)	72 (70)
Golgi_2	131	65	62 (61)	55 (54)	29 (28)
candelabrum	162	2	6	4	0
globular	90	51	4	3	3
Lugaro	59	44	30	28	22
Mli1_1	1409	6	63	47	0
Mli1_2	3003	23	140	82	2
Mli2	1369	7	63	45	0

Table legend: Cutoff levels for gene detection were set to zero unless otherwise indicated. Values in parenthesis give numbers of Glra positive cells if the cutoff for Glra was set to 0.3

(see main text for details), “Glra” stands for any of the Glra1, 2 or 3 receptor subunits. Glra4 was not expressed in the data analyzed.

As may be taken from this table, there are two major groups of nuclear GABAergic and/or glycinergic interneurons, in clusters i3 and i2_1, that express *Glra* subunits and subunit *Glrb*, i.e. are conceivably capable to express functional glycine receptors [21]. This holds for some 71 % of all i3 cells, and about 30 % of cells in cluster i2_1. Potentially glycine-sensitive cells in these clusters also differ by levels of *Glra* mRNA expression, which is much higher in i3 cells than in those of cluster i2_1 **(Fig. 3)**. Thus, average expression levels for *Glra1, 2* and *3* were ~ 6, 60, and 300 times higher in i3 than in i2_1 cells. Yet as expression values were not normally distributed, it may also be sensible to check for potential highly expressing outliers. If we focus on *Slc6a5*-positive cells and neglect, for the moment, cells with low expression of *Glra* expression (less than 0.3; average expression in i3 ~ 0.5, 1.6 and 0.9 of *Glra1, 2* and *3*, respectively), this difference becomes even more striking: thus, 55% of i3 cells (absolute number, 52), but only 3.8% of i2_1 cells (21 cells) meet these criteria, i.e. are both glycinergic and express substantial levels of *Glra* receptors, comparable to the levels also seen in Golgi cells.

Glycinergic (*Slc6a5*)-positive cells in clusters i3 and i2_1 not only differ by the levels of *Glra* subunits they express. A comparison of these groups also revealed highly significant differences in the expression of, inter alia, mRNAs coding for the adhesion molecules *Pcdh9, Pcdh15,* and *Dcc,* the calcium binding protein *Necab1*, the transmitter receptor subunits *Grik2* and *Gabarg3*, the microtubule binding protein, *Nckap5* , the zinc-finger transcription factor, *Zeb2* (all higher in i3 cells), or the adhesion molecules *Cdh18* and *Cdh22*, the actin associated protein *Whrn,* the protein tyrosine phosphatase receptor P*tprz1* and the phosphodiesterase *Pde4b* (all higher in i2_1 cells), to name but a few examples drawn from the 60 most differentially expressed genes.

Discussion

We integrated and compared gene expression data from the cortex and nuclei of adult murine cerebella with the principal goal to further characterize nuclear GABAergic and mixed GABAergic/glycinergic neurons. The results reported indicate that the molecular diversity of Purkinje neurons as revealed by their differential expression of zebrin II may not be translated into a comparable subdivision of nucleo-olivary projection neurons. Gene expression patterns of nuclear inhibitory interneurons were found to resemble those seen in inhibitory interneurons resident in the granule cell and Purkinje cell layers. Lastly, the present analysis suggests a rationale for the curious intrinsic electrophysiological silence, in slice preparations, of that subset of nuclear (inter-) neurons that also project to the cerebellar cortex [39].

Limitations

An obvious limitation of this study is that any functional interpretation presupposes that mRNA expression is indicative of the expression of functional proteins. Even if so, the data analyzed do not give any information about differential splicing,

posttranscriptional editing, let alone posttranslational modifications, all of which are known to significantly affect neural physiology (e.g., [7, 9, 11, 34]). However, while the findings reported do not inform about this post-transcriptional level of functional complexity, at a minimum they may help to focus future research.

Another point to keep in mind is that the samples analyzed comprise about 11% of all nuclear GABAergic and/or glycinergic cells (2363 of some 21500 cells; [2, 38]), and even a lower percentage of all cortical cells (~ 2.6%; estimated based on Purkinje cell numbers, cf [40]). This may arguably affect the precision of numerical estimates for smaller neuronal sets reported here.

Projection neurons

Cells in nuclear cluster i1, which comes to lie close to Purkinje cells in UMAP projection, are characterized by the differential expression of several RNAs, including that of *Dmbx1*.

During cerebellar development, *Dmbx1* is also expressed in at least a subset of cells positive for *Sox14* (KS, unpublished), a transiently expressed marker for a subset of nucleo-olivary projection neurons essential for their differentiation [28]. Together, these observations support the suggestion by [15] that this cluster comprises nucleo-olivary projection neurons.

Interestingly, while i1 cells form a rather tight cluster, they are also characterized by the differential expression of a sizable set of genes, including *Dmbx1* (**Fig. 1B**). Yet the expression pattern of none of these genes suggests a subdivision of i1 cells comparable to that of Purkinje cells revealed by expression of zebrin II (*Aldoc*). The significance, if any, of this heterogeneity for the function of the nucleo-olivary projection remains obscure. A so far unexplored issue is whether i1 cells also comprise neurons that project (also) to non-olivary targets of inhibitory nuclear neurons which have recently been described [4, 13, 20]. Unfortunately, no functional or molecular characteristics that would allow the alignment of the inhibitory, extra-cerebellar-projecting cells reported by Cao et al. [4] and Judd et al. [13] with the genetically defined subsets of cerebellar inhibitory neurons as defined by Kebschull et al. [15] have been reported so far. We cannot add to the characterization of D1 receptor-positive projection neurons reported by Locke et al. [20], as only nine cells expressing the mRNA for this receptor are present in the data for nuclear cells available, spread out across the inhibitory clusters identified.

Local inhibitory interneurons – and their not so local brethren

While the nuclear cells in clusters i2 (and all of its subclusters) and i3 are clearly separated from nucleo-olivary projection neurons and come to lie next to cerebellar cortical inhibitory interneurons in UMAP projections (and also when projected using tSNE), their global classification as “nuclear inhibitory interneurons” falls somewhat short of reality. Thus, Uusisaari and Knöpfel [39] and Ankri et al. [1] established that a subset of *Slc6a5*-positive nuclear neurons (also) projects back to the cerebellar cortex, where they innervate a *Slc6a5*-negative subset of Golgi neurons. It has been suggested that these inhibitory (GABA- and glycinergic) projections arise from cells of cluster i3 [14]. The genetic similarity / proximity of i3 cells to Lugaro neurons supports this view, as Lugaro cells also target Golgi cells [6] (for further references and discussion,

see also [33]). A somewhat anecdotal support for this view is further suggested by the observation that Lugaro cell axons – in contrast to the axons of all other cortical interneurons – have been observed to pass, occasionally, through the cerebellar white matter [17]. Yet the most intriguing argument in support of this view builds on the observation that these cells are strongly positive for mRNAs that predict the expression of functional glycine receptors. Uusisaari and Knöpfel [39] noted that the nuclear *Slc6a5*-positive cells projecting to the cortex, when analyzed in acute slices, did not show spontaneous activity and thereby could be functionally distinguished from local interneurons positive for *Slc6a5*. They also observed that blockade of GABA-receptors in the slices did not induce these cells to become active. The present findings suggest that it may not be GABAergic, but rather glycinergic input that suppressed spontaneous activity of the *Slc6a5*-positive nuclear neurons projecting to the cortex. The data reported here also reveal that i3 cells are the only inhibitory neuronal subgroup expressing substantial levels of mRNAs predicting the expression of functional glycine receptors (**Fig. 3**).

While we cannot exclude that cells from cluster i2_1 are also sensitive to glycine, the low levels of mRNAs for alpha subunits of the glycine receptor that these cells express suggests that this is a more remote possibility. More generally, differential expression of the mRNAs for glycine receptors suggests that cells of cluster i3 are the only population of inhibitory interneurons sensitive to glycine, and that i1 inhibitory interneurons may also differ in their sensitivity to glycine (**Fig. 1B**). In contrast, glycinergic cells are found in clusters i3 and i2_1, and rarely also in cluster i2_2. No *Slc6a5*-positive cells are found in cluster i2_3, which parallels the selective GABAergic nature candelabrum [27] and Mli2 cells, to which i2_3 cells resemble based on their position in UMAP-projections following clustering (**Fig. 2**).

Finally, a comment on the classification of the *Slc6a5*-positive nuclear cells that also target the cerebellar cortex identified by Uusisaari and Knöpfel [39] may be allowed. Clearly, these are no pure interneurons, with which they are usually classified, and which they very much resemble given their transcriptome. Yet it might be recalled that at least since the days of Birdsey Renshaw, it is appreciated that projection neurons also target local cells [29]. Are the cortically projecting nuclear cells of Uusisaari and Knöpfel [39] an example of local interneurons also targeting distant cells? It may be hoped that the distinct developmental history of classical projection neurons and interneurons in the cerebellum provide a paradigm to further study this blurry issue.

Conclusions

The present analysis reveals gene expression-based similarities and differences between sets to inhibitory (GABAergic and mixed GABAergic / glycinergic) cells of the cerebellar nuclei and cortex that hopefully allow focussing future research aimed at unravelling the molecular characteristics and identity, wiring and functional significance of the cells of the cerebellar nuclei.

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