Single-Cell Transcriptomics Reveals Regulators of Neuronal Migration and Maturation During Brain Development

Journal of Experimental Neuroscience
Volume 12: 1–4

DOI: 10.1177/1179069518760783

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ABSTRACT: The correct establishment of inhibitory circuits is crucial for cortical functionality and defects during the development of γ-aminobutyric acid–expressing cortical interneurons contribute to the pathophysiology of psychiatric disorders. A critical developmental step is the migration of cortical interneurons from their site of origin within the subpallium to the cerebral cortex, orchestrated by intrinsic and extrinsic signals. In addition to genetic networks, epigenetic mechanisms such as DNA methylation by DNA methyltransferases (DNMTs) are suggested to drive stage-specific gene expression underlying developmental processes. The mosaic structure of the interneuron generating domains producing a variety of interneurons for diverse destinations complicates research on regulatory instances of cortical interneuron migration. To this end, we performed single-cell transcriptome analysis revealing *Dnmt1* expression in subsets of migrating interneurons. We found that DNMT1 preserves the migratory morphology in part through transcriptional control over *Pak6* that promotes neurite complexity in postmigratory cells. In addition, we identified *Ccdc184*, a gene of unknown function, to be highly expressed in postmitotic interneurons. Single-cell mRNA sequencing revealed a positive correlation of *Ccdc184* with cell adhesion–associated genes pointing to potential implications of CCDC184 in processes relying on cell-cell adhesion–like migration or morphological differentiation of interneurons that deserves further investigations.

KEYWORDS: Single-cell transcriptomics, migration, maturation, interneurons, DNMT, Pak6, Ccdc184, epigenetics

RECEIVED: January 31, 2017. **ACCEPTED:** February 1, 2018.

TYPE: Commentary

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The research was funded by DFG and EKFS.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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COMMENT ON: Pensold D, Symmank J, Hahn A, Lingner T, Salinas-Riester G, Downie BR, Ludewig F, Rotzsch A, Haag N, Andreas N, Schubert K, Hübner CA, Pieler T, Zimmer G. The DNA Methyltransferase 1 (DNMT1) Controls the Shape and Dynamics of Migrating POA-Derived Interneurons Fated for the Murine Cerebral Cortex. Cereb Cortex. 2017 Dec 1;27(12):5696-5714. doi: 10.1093/cercor/bhw341. PubMed PMID: 29117290.

In the cerebral cortex, the seat of higher cognitive functions, neuronal circuitries are composed of excitatory glutamatergic principal neurons and inhibitory γ-aminobutyric acid (GABA)-expressing interneurons. Although representing only 10% to 20% of the overall neuronal population, the inhibitory GABAergic interneurons are essential key players in cortical information processing shaping the excitatory responses of principal cells. Developmental defects leading to altered cortical interneuron numbers, and hence a disturbed balance of excitation and inhibition, contribute to the pathophysiology of diverse neurologic and psychiatric diseases.[1,2] Therefore, decoding the regulatory instances required for the generation and maturation of cortical interneurons attracts attention to the field and holds great promise for the development of cell replacement strategies.[3]

In contrast to principal neurons, which arise from the cortical proliferative zones,[1] GABAergic cortical interneurons are born in different domains of the basal telencephalon, including the medial and caudal ganglionic eminences (MGE and CGE, respectively) as well as the preoptic area (POA).[1] Newborn cortical interneurons adopt a migratory morphology[1] that enables tangential migration along particular routes through the subpallium up to the cortex.[4,5] After entering the cortex, they spread across different cortical areas before radially invading their target layers and forming their synaptic network.[1] These distinct steps of maturation emphasize the relevance of postmitotic regulatory instances in addition to the well-documented significance of progenitor information[1,3] exerting control over interneuron migration and maturation.

To reveal potential key players driving cortical interneuron migration, we performed single cell–based transcriptome analysis of cells prepared from the POA domain, as this mosaic tissue simultaneously generates migratory active interneurons destined for distant brain regions such as the cortex or the striatum as well as residual neurons differentiating on-site within the mantle of the POA.[6,7] The striatal-fated cells characterized by *Isl1* expression, a marker of striatal neurons, emerged as 1 of 2 main subsets of POA-derived GABAergic cells as revealed by non-supervised cluster analysis. The second large cohort of POA-derived GABAergic neurons displayed prominent *Pak6* expression, which was evident in about 79% of the cells collected in this cluster. PAK6 is a member of the family of p21-activated kinases (PAKs) known to promote neurite outgrowth of postmigratory cells.[8] Indeed, by manipulation of *Pak6* expression levels in embryonic POA-derived cells, we confirmed that PAK6 drives their morphological differentiation and maturation.[6] The PAKs are well-known effector proteins of the Rho GTPases, Rac and Cdc42, that are essential for cytoskeletal organization underlying cell shape, motility and adhesion as central key players of actin dynamics.[8] For the group-B PAKs, which consist of PAK4, PAK5 and PAK6, a major role in filopodia formation downstream of Cdc42 was reported.[8] A similar function like we found for PAK6 promoting neurite outgrowth in POA cells has been described by

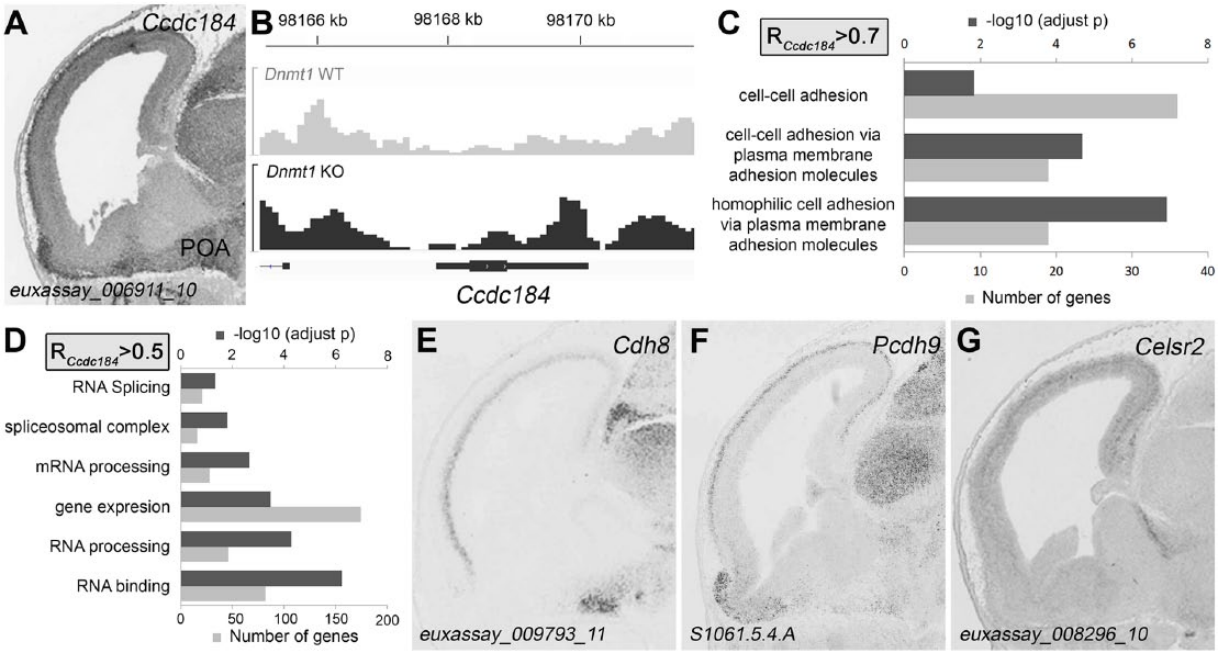

Figure 1. *Ccdc184* is associated with cadherin-based cell-cell adhesion processes in POA-derived interneurons. (A) *In situ* hybridization against *Ccdc184* in E14.5 sagittal sections of C57/Bl6 mice shows expression in the ventral part of the basal telencephalon and especially in the preoptic area (POA). (B) The methylation density of the *Ccdc184* locus is not reduced in *Dnmt1*-deficient *Hmx3-Cre/tdTomato* cells compared to wild-type as revealed by MeDIP (methylated DNA immunoprecipitation) sequencing. (C, D) Gene Ontology (GO) enrichment analysis of genes found significantly correlated with *Ccdc184* ($R > 0.7$ in C) in POA-derived single cells revealed an enrichment of cell-cell adhesion–associated genes. Further terms related to RNA processing and gene expression appear when expanding the correlated genes to a correlation coefficient of (D) $R > 0.5$. (E-G) In situ hybridization against (E) *Cdh8*, (F) Pcdh9, and (G) Celsr2 in E14.5 sagittal sections of C57/Bl6 mice showing expression of these cadherins in the POA. In situ hybridizations were obtained from Gene Expression Database (GXD) or genepaint.org; accession numbers are provided in each panel. Data analysis was performed as described in the work by Pensold et al., 2017.

Cobos et al.[9] for PAK3 in postmigratory MGE-derived interneurons. During the migratory period of MGE interneurons, *Pak3* is repressed by *Dlx1,2* to maintain the migratory morphology.[9]

The single-cell resolution revealed differing levels of *Pak6* expression for the cells collected in this POA cell cluster, ranging from high over medium to no expression. Correlation analysis revealed that *Pak6* negatively correlates with *Dnmt1* expression in embryonic POA-derived cells, which was detected in some but not all of these POA cells.[6] As PAK6 seemed crucial for postmigratory processes, we hypothesized that *Dnmt1* expression was maintained in a subset of migratory active postmitotic POA-derived GABAergic interneurons. Due to its function in regulating gene expression through DNA methylation–dependent and independent actions,[10,11] DNMT1 was an attractive candidate as key regulator of POA interneuron migration. Evidence for a crucial function of DNMT1 in regulating cortical interneuron development came from studies of Matrisciano et al.[12] showing that prenatally stress-induced changes in *Dnmt1* expression levels in embryonic murine GABAergic interneurons elicited schizophrenia-like phenotypes in adult offsprings. In addition, it has been reported that dysregulated *Dnmt1* expression and DNMT1 action in cortical GABAergic interneurons are implicated in the pathophysiology of several psychiatric diseases.[2,13]

To examine the specific function of DNMT1 in the context of POA interneuron development, we conditionally deleted *Dnmt1* in POA-specific *Hmx3*-expressing postmitotic cells.[6] Although the number of residual POA-fated cells was unaffected, the fraction of interneurons reaching the cortex was severely diminished in adult mice, similar to what was reported for postmortem brains of patients with schizophrenia.[2,13] This reduction in interneuron number was caused by migratory defects due to morphological abnormalities of *Dnmt1*-deficient interneurons during embryonic development.[6] The morphological defects led to a reduced migratory capacity of POA-derived cortical interneurons followed by neuronal degeneration and increased cell death.[6] DNMT1 was already described to be critical for postmitotic maturation and survival of other neuronal subtypes *in vitro* and *in vivo*.[14,15]

Next, we asked for the underlying mechanism by which DNMT1 controls the migratory morphology and performance of cortical interneurons. Canonical DNMT1 function often leads to cytosine methylation mostly associated with gene silencing.[10,11] The migratory morphology largely depends on cytoskeletal remodeling executed by associated and regulating proteins.[7] Accordingly, we observed correlated transcriptome and methylome changes in genes related to cytoskeleton organization and cell morphogenesis applying MeDIP (methylated DNA immunoprecipitation) and RNA sequencing of

embryonic fluorescence-activated cell–sorted *Hmx3-Cre/tdTomato/Dnmt1* wild-type and knockout cells.[6] Hence, DNMT1 could repress the transcription of genes involved in cytoskeleton rearrangements underlying the adoption of a differentiating morphology including neurite outgrowth and branching as we showed for PAK6.[6]

In agreement with the negative correlation between *Dnmt1* and *Pak6* at single-cell level, *Pak6* expression was found increased in *Dnmt1*-deficient cells. However, we did not observe differential methylation levels in the *Pak6* gene locus, neither in regulatory regions nor within the gene body.[6] Also, direct potential regulators of *Pak6* with predicted binding motifs were not changed in their expression or methylation.[6] This points to a noncanonical regulation of *Pak6* expression through DNMT1 by acting on repressive histone modifications which are investigated in ongoing studies (data not shown).

In addition to DNMT1 driving migration and PAK6 promoting morphological differentiation, the single-cell transcriptomics presented in the work by Pensold et al.[6] revealed another interesting gene as significantly expressed in postmitotic POA-derived cells that remained undiscussed so far. We found the gene *Ccdc184*, which is a coiled-coil domain protein of unknown function, significantly enriched within the *Pak6*-positive cell cluster.[6] The expression in POA-derived cells was confirmed by in situ hybridization (Figure 1A)[6] as well as by RNA sequencing of MGE and POA tissue, showing a significantly elevated expression in POA samples (fold change$_{log2}$ = 2.62 upregulated in POA tissue compared with MGE; $^{***}P_{adj}$ = 9.77E−66).[6] *Ccdc184* displayed a random expression correlation with *Dnmt1* (R_{Person} = −0.09, P_{adj} = 0.73) at single-cell level, indicating that *Ccdc184* is regulated independently of DNMT1. This is further supported by the finding that no differential expression was detected in *Hmx3-Cre/tdTomato/Dnmt1* knockout cells compared with wild types as determined by RNA sequencing of fluorescence-activated cell sorting–enriched cells (fold change$_{log2}$ = 0.42; P_{adj} = 0.21). Consistently, we did not detect significant changes in the methylation levels for *Ccdc184* in *Hmx3-Cre/tdTomato/Dnmt1* knockout samples (Figure 1B).

Correlation studies in combination with Gene Ontology (GO) analysis enable predictions about potential gene functions. Therefore, we performed GO analysis of genes that were positively correlating with *Ccdc184* (n = 338, $R > 0.7$, $P_{adj} < 0.05$) in POA-derived single cells, and we revealed an enrichment of genes associated to homophilic cell adhesion via plasma membrane adhesion molecules (Figure 1C). Within this term, cadherins and their interaction partners were particularly enriched in our data set (eg, *Cdh8, Pcdh9, Pcdha11, Celsr2, Pkd1, Crebbp*). In situ hybridization data obtained from the mouse Gene Expression Database (GXD)[16] confirmed the expression of *Cdh8, Pcdh9*, and *Celsr2* in the POA (Figure 1F–G).

Cadherins are glycosylated transmembrane proteins regulating morphogenesis by mediating cell-cell adhesion and are involved in intracellular signaling pathways.[17,18] Hence, CCDC184 could be implicated in subcellular processes underlying cell adhesion, which is crucial for neuronal migration as well as morphological differentiation, including neurite as well as axonal outgrowth and the establishment of synaptic contacts.[1,17,18] Cadherins are involved in both, guided neuronal migration as well as the establishment of dendritic arborization, synaptic formation and remodeling.[17,18] For several cadherin complexes, it is already known that they interact with different coiled-coil proteins.[19,20] Thus, *Ccdc184* could acts as a direct interaction partner for cadherins and protocadherins during cell adhesion events. DIPA family members (Ccdc85a, Ccdc85b/DIPA, and Ccdc85c), which are common coiled-coil domain proteins, were already described to interact selectively with p120 family members capable of recruiting DIPA to adherent junction–associated cadherin complexes. Alterations in Delta-Interacting Protein A (DIPA) recruitment result in a neural tube defect similar to that caused by N-cadherin mutation in zebrafish, supporting the importance of these coiled-coil domain protein-cadherin interactions.[19] Moreover, Liu et al. showed that the coiled-coil protein SNX16 is necessary for correct recycling and trafficking of E-cadherin.[21]

Interestingly, GO analysis of genes that positively correlated to 50% with *Ccdc184* expression levels (n = 555, $R > 0.5$, $P_{adj} < 0.05$) resulted in significant GO terms related to gene expression and RNA processing (Figure 1D). This is consistent with the described functions of many coiled-coil–type proteins in the regulation of gene expression acting as transcription factors.[22] Hence, in addition to its potential role as protein interaction partner, *Ccdc184* could function as transcriptional regulator controlling the cell-specific expression of genes coding for cell adhesion proteins such as cadherins or other cell-cell adhesion–associated genes. However, detailed functional analysis is required to determine the particular functions of the coiled-coil domain protein CCDC184.

Together, in addition to validated genes, the single-cell transcriptomics presented in the study by Pensold et al.[6] revealed interesting candidate genes of yet unknown function such as *Ccdc184* that are potentially involved in interneuron development and maturation, which deserves further investigations.

Author Contributions

DP performed data analysis, figure illustration and wrote the manuscript. GZ wrote the manuscript.

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