The transcription factor BCL11A defines distinct subsets of midbrain dopaminergic neurons

Highlights

- Bcl11a is expressed in a subset of murine and human dopaminergic neurons
- Bcl11a<sup>+</sup> dopaminergic neurons form a highly specific subcircuit in mouse
- Bcl11a<sup>+</sup> substantia nigra neurons are particularly vulnerable to neurodegeneration
- Bcl11a inactivation increases vulnerability and impairs motor behavior in mice

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In brief

Little is known about the molecular mechanisms that establish diversity in the dopaminergic system. Tolve et al. show that the transcription factor BCL11A defines dopaminergic subpopulations with distinct projection targets and susceptibility to neurodegeneration and is required for the establishment and maintenance of normal function of this subset.
Article

The transcription factor BCL11A defines distinct subsets of midbrain dopaminergic neurons

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SUMMARY

Midbrain dopaminergic (mDA) neurons are diverse in their projection targets, effect on behavior, and susceptibility to neurodegeneration. Little is known about the molecular mechanisms establishing this diversity during development. We show that the transcription factor BCL11A is expressed in a subset of mDA neurons in the developing and adult murine brain and in a subpopulation of pluripotent-stem-cell-derived human mDA neurons. By combining intersectional labeling and viral-mediated tracing, we demonstrate that Bcl11a-expressing mDA neurons form a highly specific subcircuit within the murine dopaminergic system. In the substantia nigra, the Bcl11a-expressing mDA subset is particularly vulnerable to neurodegeneration upon α-synuclein overexpression or oxidative stress. Inactivation of Bcl11a in murine mDA neurons increases this susceptibility further, alters the distribution of mDA neurons, and results in deficits in skilled motor behavior. In summary, BCL11A defines mDA subpopulations with highly distinctive characteristics and is required for establishing and maintaining their normal physiology.

INTRODUCTION

Midbrain dopaminergic (mDA) neurons are anatomically organized into the substantia nigra (SN), ventral tegmental area (VTA), and retro-rubral field (RRF). These anatomically defined areas contain subpopulations of mDA neurons that are characterized by distinct connectivity and distinct effects on behavior (Cox and Witten, 2019; Engelhard et al., 2019; Morales and Margolis, 2017). The SN consists of the pars compacta (SNc), containing most SN-mDA neurons; the pars lateralis (SNl); and the pars reticularis (SNr). SNl mDA neurons project to the tail of the striatum (TS) (Menegas et al., 2015). Medial SNc mDA neurons send their axons to the dorsomedial striatum (DMS), and projections of the lateral SNc target the dorsolateral striatum (DLS) (Lerner et al., 2015). mDA neurons in the VTA project to the nucleus accumbens (NAC), the olfactory tubercle (OT), prefrontal cortex (PFC), and amygdala. The VTA is also anatomically divided into smaller domains, but the relationship between the anatomical location of mDA cell bodies and their projection targets is less well defined (Morales and Margolis, 2017).

In addition to these anatomical mDA subgroups, mDA neurons have been classified based on their molecular profile. A comparison of the currently available single-cell gene expression studies led to the proposal that there are at least 7 molecularly defined mDA subgroups. These subgroups do not necessarily correspond to mDA populations distinct by anatomical location, projection target, and functional output (Poulin et al., 2020), and thus, it is not clear to what extent these different levels of diversity can be reduced to a common denominator to define mDA diversity. Because anatomical position, molecular profile, and...
connectivity are largely determined during development, a better understanding of the developmental factors that determine mDA subpopulations could deliver important new insights on how to define mDA subpopulations.

mDA neurons in the SNc degenerate in Parkinson’s disease (PD), leading to the cardinal motor symptoms of PD. mDA neurons in the VTA are much less affected by the neurodegeneration in PD and in various PD models in rodents, but even within the SNc, neurons are not homogeneous in their vulnerability: SNc-mDA neurons in the ventral tier appear to be more vulnerable than the ones in the dorsal tier (Surmeier et al., 2017). In mouse, ventral tier neurons express ALDH1A1 (Aldehyde Dehydrogenase 1 Family Member A1), whereas the less vulnerable subpopulation in the dorsal tier expresses the Calcium-binding protein Calbindin1 (CALB1) (Liu et al., 2014; Poulin et al., 2014; Thompson et al., 2005; Wu et al., 2019), suggesting that the susceptibility to neurodegeneration in mDA subgroups could be genetically and developmentally pre-determined.

Because transcription factors are key regulators of cell specification programs, we set out to identify transcription factors that are expressed in subpopulations of mDA neurons in the developing and adult brain. We discovered that the C2H2 zinc finger transcription factor BCL11A (B cell CLL/lymphoma 11A) is expressed in a subpopulation of mDA neurons from embryogenesis to adulthood. BCL11A is a transcriptional repressor and a dedicated subunit of the mammalian SWI/SNF complex, which functions as an ATP-dependent chromatin remodeler (Kadoch et al., 2013; Simon et al., 2020). In mouse, BCL11A regulates neuronal fate determination during cortex and spinal cord development (Simon et al., 2020). In humans, BCL11A haploinsufficiency results in neurodevelopmental disorders characterized by developmental delay, intellectual disability, and behavioral problems (Basak et al., 2015; Dias et al., 2016; Fitzgerald et al., 2015; Peron et al., 2019; Soblet et al., 2018). Although these studies point to the importance of BCL11A in the development of the central nervous system (CNS), the function of BCL11A in the development and maintenance of the dopaminergic (DA) system has not yet been examined.

RESULTS

BCL11A is expressed in a subset of mDA neurons in the mouse brain

We initially identified Bcl11a as a potential mDA-subset marker based on the expression pattern available on the Allen Brain Atlas (Developing Mouse Brain). To examine whether BCL11A/Bcl11a is indeed expressed in a subpopulation of mDA neurons, we analyzed the expression of Bcl11a mRNA and BCL11A protein in combination with tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis. In the neonatal and postnatal day 30 (P30) brain, Bcl11a mRNA was expressed in a subset of SN, VTA, RRF, and caudal linear nucleus (CLi) neurons. In the SN, Bcl11a-expressing mDA neurons were predominantly localized to the SNi and the dorsal SNc. Within the VTA, Bcl11a-positive neurons were found primarily in the lateral VTA (Figures 1A–1F; data not shown). The distribution of neurons positive for BCL11A protein and TH was comparable to the distribution of Bcl11a/TH-positive neurons in the neonatal brain and in the VTA of P30 brains (Figures 1G–1L). However, we could not detect the BCL11A protein in mDA neurons in the SN at P30 (Figures 1J and 1L). Bcl11a mRNA is expressed at lower levels in SN than in VTA-mDA neurons at P30 (Figures 1E and 1F), indicating that SN-mDA neurons may express the BCL11A protein at low levels that are undetectable with the anti-BCL11A antibody used in our study.

Next, we analyzed the distribution of Bcl11a-expressing cells by using Bcl11alacZ mice. In this mouse line, the lacZ allele is knocked into the endogenous Bcl11a locus and β-gal expression is restricted to cells that express Bcl11a (Dias et al., 2016). Indeed, the distribution of β-gal-positive mDA neurons in neonatal and adult brain was comparable to the one of mDA neurons expressing Bcl11a mRNA (Figures 1A–1F and 1M–1R; Figure S1). Double labeling for β-gal and the BCL11A protein showed that the expression pattern of β-gal and BCL11A was largely overlapping in the VTA and in the cerebral cortex, indicating that β-gal reliably marks BCL11A-expressing neurons (Figures S2A–S2D). Quantification of β-gal and TH double-positive cells in VTA, SN, RRF, and CLi of neonatal and P30 Bcl11a-lacZ mice demonstrated that about one-third of mDA neurons express Bcl11a (Figures 1S and 1T; Figure S1G). Additionally, non-mDA neurons expressing β-gal were present medial to the VTA, dorsal to the SN, and within the VTA and SN area (Figures 1M and 1P; Figure S1).

An analysis of the developmental time course of Bcl11a expression showed that Bcl11a was first expressed in the ventral midbrain at embryonic day 12.5 (E12.5). At E12.5 and E13.5, Bcl11a was mainly localized in the area just below the mDA progenitor domain and in a few differentiated TH-expressing mDA neurons. At E14.5 and E15.5, expression was found in a larger subset of mDA neurons in the forming SN and VTA (Figures S2E–S2I; data not shown).
BCL11A-expressing human-stem-cell-derived mDA neurons show a distinct gene expression profile

To examine whether BCL11A is expressed in human mDA neurons, we analyzed data obtained from droplet-based single-cell RNA sequencing (scRNA-seq) of human induced pluripotent stem cell (hiPSC)-derived mDA neurons. These cultures contain multiple neuronal subtypes, including SNc-like (DAn1) and VTA-like (DAn4) mDA neurons as well as progenitor cells (Prog1 and Prog2) and neurons with characteristics that do not fully recapitulate in vivo mDA neuronal types (DAn2 and DAn3) (Figure 2A; Fernandes et al., 2020). DAn4 neurons were characterized by the combined expression of CALB1, MARCK5, and NEFM. DAn1 neurons were defined by the expression of TH, NR4A2, FOXA2, LMX1A, DDC, and LMO3 and the absence of serotonergic markers (TPH1). In these cultures, BCL11A single-cell detection rates suggested some cell-type specificity with the most prominent expression in DAn4 neurons followed by expression in DAn1 neurons and a progenitor-like population (Prog2) (Figures 2A–2C).

**Figure 2.** BCL11A-expressing hiPSC-derived mDA neurons show a distinct gene expression profile

(A and B) Low-dimension uniform manifold approximation and projection (UMAP) of single-cell transcriptomes. Culture of 2,648 hiPSC-derived human mDA neurons annotated by cell type (as in Fernandes et al., 2020) (A) and normalized for BCL11A expression (B). (C) BCL11A expression levels (counts per million) detected in single cells aggregated by cell type. Error bars indicate 95% confidence intervals. (D and E) Gene set enrichment analysis of the top 100 upregulated genes in BCL11A-positive DAn1 (D) or BCL11A-positive DAn4 (E) cells sorted according to statistical significance. See also Tables S1 and S2. For subtype characterization in mouse see Figure S3.
We then investigated whether BCL11A-expressing mDA neurons show a distinct transcriptomic signature (Fernandes et al., 2020). Due to the high dropout rates of BCL11A, we were constrained to analyses between BCL11A-positive cells and BCL11A-negative cells in DAn1 (SNc-like) and DAn4 (VTA-like) cell types. A differential expression analysis of 162 positive and 1,107 negative DAn1 cells using a p value threshold of 0.05 highlighted 257 upregulated and 301 downregulated genes in the DAn1 population. BCL11A expression in DAn4 cells was found to be broader, with 81 positive and 276 negative cells with differential expression analysis identifying 167 upregulated and 70 downregulated genes. We then performed a gene set enrichment analysis of the 100 most significant upregulated genes in DAn1 and DAn4 BCL11A-positive cells. This analysis highlighted changes in cell projection morphogenesis, synaptic transmission, and synapse organization pathways for DAn1 BCL11A-expressing cells (Figure 2D; Table S1). In DAn4 BCL11A-positive cells, the analysis revealed prominent changes in translation (Figure 2E; Table S2). Taken together, BCL11A defines a distinct molecular signature within SNc-like (DAn1) and VTA-like (DAn4) hiPSC-derived mDA neurons. In DAn1 neurons, this gene expression profile suggests that neuronal maturation and function of BCL11A-expressing neurons may differ from BCL11A-negative DAn1 neurons.

Based on the differential expression analysis in hiPSC-derived mDA, BCL11A-expressing neurons do not fall into any of the previously characterized mDA subclasses (Poulin et al., 2020). To examine this in the mouse brain, we focused on the mDA subpopulations expressing ALDH1A1, CALB1, and SOX6 (SRY-Box Transcription Factor 6), ALDH1A1 is expressed in the ventral VTA, whereas CALB1 shows widespread expression in the VTA (Thompson et al., 2005; Wu et al., 2019). SOX6 expression is restricted to SN-mDA neurons and to neurons of the lateral SNc (Panman et al., 2014; Poulin et al., 2018). We performed triple immunostaining for β-gal, TH, and the respective subset marker (ALDH1A1, CALB1, or SOX6) in neonatal and P30 brain of Bcl11a-lacZ mice (Figures S3A–S3D). Quantification of triple-labeled mDA neurons (Figures S3E–S3H) showed that Bcl11a-expressing mDA neurons did not fully overlap with any of these markers, confirming that Bcl11a-positive mDA neurons do not clearly fall within one of these mDA subpopulations.

Bcl11a-expressing mDA neurons form a subcircuit in the DA system

If Bcl11a-expressing neurons indeed constitute a subset in the DA system, they should feature not only a specific molecular profile but also specific connectivity patterns. To examine whether the Bcl11a-expressing subclass of mDA neurons contributes to specific subcircuits in the mDA system, we specifically labeled cell bodies and projections of Bcl11a-expressing mDA neurons by using an intersectional genetic approach. This method is based on a reporter construct in which expression of a fluorescent protein is driven by a tetracycline response element (TRE) in a Cre-dependent manner (Madsen et al., 2015; Poulin et al., 2018). We first combined the intersectional reporter mouse line Ai82D (Madsen et al., 2015) with a DAtTAA (tetracycline trans-activator driven by the DAt promoter) and a Bcl11aCreERT2 mouse line (Chen et al., 2015; Poulin et al., 2018; Pensa et al., 2021; Figure 3A). Because BCL11A expression is already restricted to mDA subsets during embryogenesis (Figures S2F–S2I), CreER was activated by administering tamoxifen to pregnant females at E15.5 (Figure 3B). Distribution of the recombinated (EGFP+) mDA neurons was comparable to the distribution of β-gal-expressing cells in Bcl11a-lacZ mice (Figures 3C, S1, and S4). An analysis of EGFP-positive fibers showed that in target areas of VTA-mDA neurons (Morales and Margolis, 2017), Bcl11a-expressing mDA neurons strongly innervated the lateral septum and the OT and the ventral and lateral shell of the NAc, but not the medial shell or core (Figures 3D and 3E; data not shown). Hardly any EGFP-positive fibers were observed in the amygdala, habenula, or PFC (data not shown). Moreover, Bcl11a-expressing mDA neurons showed a highly specific projection pattern to the TS and the caudal DMS (Figures 3D and 3E), which are target areas of mDA neurons in the SN and the medial SNc, respectively (Lerner et al., 2015; Menegas et al., 2015; Poulin et al., 2018). Within these SN target areas, the densest innervation originating from recombined mDA neurons was observed in the ventral TS. Innervation of the dorsolateral and rostral striatum was very sparse.

Next, we investigated the projections of mDA neurons that express Bcl11a in the adult brain (1) to clarify whether Bcl11a expression defines the same subset of mDA neurons in the embryonic and adult brain and (2) to clearly delineate the projection targets of Bcl11a-expressing neurons in the SN versus in the VTA. We introduced reporter constructs by injecting recombinant adeno-associated viruses (rAAVs) into the SN or VTA of adult animals. CreER was subsequently activated by the administration of tamoxifen to achieve recombination (Figures 4A and 4B; Figures S5A and S5B). We took both an intersectional approach, in which we introduced an intersectional reporter construct into Bcl11aCreERT2, DAtTAA mice (Figures 4A and 4B), and a non-intersectional approach in which we delivered a Cre-dependent reporter construct into Bcl11aCreERT2 mice (Figures S5A and S5B). Labeling of Bcl11a-expressing mDA neurons in the SN was scarcer than that in the VTA (Figures 4C, 4D, 4F, and 4G; Figures S5C, SSD, SSG, and SSH), which is consistent with a lower percentage of Bcl11a-expressing mDA neurons in the SN than in the VTA (Figure 1T). The sparse labeling allowed us to visualize patches of innervation likely derived from single SN axons. These patches were restricted to the DMS and ventral TS, consistent with the findings obtained with the embryonically induced intersectional labeling (Figure 3D; Figures 4E and 4E′; Figures S5E and S5F). Characterization of the VTA projection targets showed strong innervation of the OT and the ventral and lateral shell of the NAc (Figures 4F–4I; Figures S5G–S5K), consistent with the innervation pattern observed with the embryonic labeling (Figure 3). In some animals (n = 6/10) with sparse labeling of Bcl11a-expressing VTA-mDA neurons, the OT was innervated in a stripe-like pattern, suggesting that axons from single neurons might target specific domains within the OT (Figure S5). In conclusion, these results show that expression of Bcl11a in embryogenesis and adulthood defines subsets of SN and VTA mDA neurons that form a highly specific subcircuit within the mDA system.
Figure 3. Bcl11a-expressing mDA neurons contribute projections to subcircuits of the dopaminergic system

(A) Intersectional fate mapping strategy to label Bcl11a-expressing mDA neurons and their projections. Bcl11a<sup>CreER</sup> mice were used in combination with Dat<sup>TTA</sup> mice and an intersectional reporter mouse line (Ai82D). EFGP is expressed only in cells positive for both CreER and tTa and only after CreER is activated by tamoxifen administration.

(B) Experimental timeline.

(C) Distribution of mDA neurons labeled with the intersectional fate mapping approach (EGFP<sup>+</sup> and TH<sup>+</sup>) for individual mice (1–7). SN: l_SNc, lateral SNc; m_SNc, medial SNc; and SNl; VTA: IF, infundibular nucleus; PBP, parabrachial nucleus; and PN, paranigral nucleus (see Figure S1F). Colored circles are matched with the colors of the projection analysis in (E).

(D) Quantification of the density of mDA axons labeled with the intersectional fate mapping approach (EGFP<sup>+</sup>) in olfactory tubercle (OT), nucleus accumbens core (NacC), nucleus accumbens shell (NacSh), rostral striatum (RS), dorsomedial striatum (DMS), dorsolateral striatum (DLS), and ventral and dorsal tail of striatum (vTS and dTS, respectively). n = 7 mice. Error bars indicate mean ± SEM.

(E) Projections of mDA axons labeled with the intersectional fate mapping approach (EGFP<sup>+</sup>) in five rostrocaudal striatal coronal sections shown for individual mice (indicated by different colors, as in C). TH-positive fibers are shown in gray. Scale bars: 500 μm.
Conditional gene inactivation of Bcl11a in mDA neurons results in altered positioning of Bcl11a-mDA neurons

Because Bcl11A is a transcription factor that has been shown to influence neuronal fate, neuronal morphology, and migration (Simon et al., 2020), we investigated whether BCL11A is necessary for establishing and/or maintaining Bcl11a-expressing mDA neurons. We generated a specific knockout for Bcl11a in mDA neurons by crossing Bcl11a<sup>fox</sup> mice with Dat<sup>RES-Cre<sup>+</sup></sup>, Bcl11<sup>cko</sup> mice (Bcl11a<sup>ckoflox</sup><sup>+</sup>; genotype: Dat<sup>RES-Cre<sup>+</sup></sup>, Bcl11a<sup>ckoflox</sup>) (Båckman et al., 2006; John et al., 2012). In a subset of mice, we introduced the Bcl11a<sup>lacZ</sup> allele (a null allele; Dias et al., 2016) into the conditional knockout model (Bcl11a<sup>cko</sup>; genotype: Dat<sup>RES-Cre<sup>+</sup></sup>, Bcl11a<sup>ckoflox</sup><sup>lacZ</sup>) (Figure 5A). In Bcl11a<sup>cko</sup><sup>lacZ</sup> mice, the Bcl11a-expressing mDA population expresses β-gal even after inactivation of Bcl11a, allowing us to analyze the effect of Bcl11a inactivation specifically in mDA neurons that would normally express BCL11A (termed Bcl11a-mDA neurons, β-gal and TH positive) (Figure 5B). We performed immunostaining and multiplex fluorescent in situ hybridization confirming that the BCL11A protein and Bcl11a mRNA expression were indeed absent in mDA neurons of Bcl11a<sup>cko</sup> mice. As expected, Bcl11a<sup>cko</sup>/Bcl11a<sup>lacZ</sup> mice was still expressed in non-mDA neurons in the midbrain and cerebral cortex (Figure 5E).

The anatomical organization of the mDA area in Bcl11a conditional knockout (cko) (both in Bcl11a<sup>cko</sup><sup>fox</sup> and Bcl11a<sup>cko</sup><sup>lacZ</sup> mice was comparable to that of controls (Figures 5G and 5D; data not shown), and the overall percentage of Bcl11a-mDA neurons (SN plus VTA) was not significantly changed between Bcl11a<sup>cko</sup><sup>fox</sup> and Bcl11<sup>lacZ</sup> mice (Figures 5E and 5F). However, a detailed analysis by anatomical area and levels revealed alterations in the distribution of Bcl11a-mDA neurons in P30 Bcl11a<sup>cko</sup><sup>lacZ</sup> mice. The percentage of Bcl11a-mDA neurons was significantly increased in the SNc and SNv of Bcl11a<sup>cko</sup><sup>lacZ</sup> mice, but in the rostral SNc, Bcl11a-mDA neurons were shifted from medial to lateral as compared to controls (Figures 5G, 5C, and 5D). These results indicate that the specific inactivation of Bcl11a in mDA neurons does not interfere with the generation or the survival of the Bcl11a-mDA neuronal population but that it results in altered positioning of Bcl11a-mDA neurons in the VTA and SNc.

If BCL11A is important for determining the localization of mDA neurons, it may also influence cell fate by regulating the expression of subset markers or the target specificity of Bcl11a-mDA projections. However, triple labeling for β-gal, TH, and CALB1 at P0 and P30; for β-gal, TH, and SOX6 at P0; and for β-gal, TH, and ALDH1A1 at P30 did not reveal a significant change in the number of Bcl11a-mDA neurons co-expressing these markers in Bcl11a<sup>cko</sup><sup>lacZ</sup> mice as compared to Bcl11a<sup>cko</sup><sup>lacZ</sup> mice (Figures S7E–S7J and S3). To assess the targeting of projections arising from Bcl11a-mDA neurons, we examined the density of TH innervation in control and Bcl11a cko mice in the ventral TS and the OT (the two areas with the highest innervation density from Bcl11a-mDA neurons) and in the dorsal striatum and the dorsal TS. We found no significant difference in the density of the TH innervation in any of these areas when comparing control and Bcl11a cko mice (Figures S7K–S7Q). These data suggest that the inactivation of Bcl11a has no overt effect on the anatomy of Bcl11a-mDA neuronal projections.

Inactivation of Bcl11a in mDA neurons results in skilled motor learning deficits

To investigate whether the specific inactivation of Bcl11a in mDA neurons leads to functional impairment in the DA system, we examined behaviors associated with VTA and/or SN function in Bcl11a cko and control mice. In the open field, distance moved was not altered in Bcl11a cko mice as compared to controls, indicating that spontaneous motor behavior was not affected in the mutant mice (Figure 6A). Decreased dopamine release from the VTA is associated with anxiety- and depressive-like behavior, which can be assessed by monitoring the activity in the center of an open field (Tye et al., 2013; El Yacoubi et al., 2003). Bcl11a cko mice did not show a change in the frequency of crossing into the center and time spent in center or border area in the open field, indicating that the Bcl11a cko mice did not have an increased level of anxiety or depressive behavior (Figures 6B and 6C). Next, we focused on tasks in which dopamine release from SN-mDA neurons is thought to play a prominent role. Dopamine release in the striatum is crucial for voluntary movement and motor skill learning (Cox and Witten, 2019; Dodson et al., 2016; Wu et al., 2019). Motor coordination and balance were examined by monitoring the ability of mice to cross a balance beam (Luong et al., 2011). We found no difference in the performance of Bcl11a cko mice and control mice in this task (Figure 6D). To examine whether motor skill learning is altered in Bcl11a cko mice, mice had to perform an accelerating rotarod test (Costa et al., 2004). In this task, control mice improved their performance over time, as reflected in a continuous increase in the time to fall over a 5-day training period. In contrast, Bcl11a cko mice did not show any improvement in their performance over time, indicating that they were not able to learn this skilled motor task within the trial period (Figure 6E). These results show that the specific inactivation of Bcl11a in mDA neurons results in deficits in skilled motor learning and suggest that Bcl11a-mDA neurons are functionally impaired in the absence of Bcl11A.
Figure 5. BCL11A is necessary for establishing the correct anatomical position of Bcl11a-expressing mDA neurons

(A) Conditional gene inactivation of Bcl11a in mDA neurons. Bcl11a cko mice were generated by crossing DatIRES-Cre mice with Bcl11aflox/flox mice (genotype: DatIRES-Cre/+, Bcl11aflox/flox, termed Bcl11a cko^flox^) or with Bcl11aflox/lacZ mice (genotype: DatIRES-Cre/+, Bcl11aflox/lacZ, termed: Bcl11a cko^flox^).

(B) In Bcl11a cko^lacZ^ mice, β-gal is a marker for Bcl11a-mDA neurons even after BCL11A expression is abolished.

(legend continued on next page)
BCL11A acts a neuroprotective factor in SNc-mDA neurons

The next set of experiments was aimed at elucidating the role of BCL11A in the context of neuronal challenges and neurodegenerative processes affecting mDA neurons in the SNc. The protein α-synuclein plays a key role in the pathogenesis of PD and other human neurodegenerative diseases (Goedert et al., 2013). Its overexpression in animal models is associated with PD-like pathology, including the degeneration of SNc-mDA neurons and accumulation of modified α-synuclein forms (Migdaelska-Richards et al., 2017; Ulusoy and Di Monte, 2013). Adult control (n = 5) and Bcl11a cko (n = 5) mice were challenged with a single intraparenchymal injection of rAAVs carrying the DNA for human α-synuclein (Figure 7A). The unilateral injection targeted the right SNc where it caused robust overexpression of human α-synuclein within SNc-mDA neurons. Immunostaining against human α-synuclein showed no difference in overexpression between control and Bcl11a cko mice (Figure 7B).

Next, TH immunoreactivity was compared in the intact and injected side of the SNc in animals sacrificed 8 weeks after rAAV injection. Labeling was overtly less robust in the lesioned SNc of both control and Bcl11a cko mice, but the reduction in TH immunoreactivity appeared to be more pronounced in Bcl11a cko animals (Figure 7C). Stereological counts in the intact SNc were similar between control and Bcl11a cko mice and were therefore averaged together as normal values (5,988 ± 79.4 cells). Overexpression of α-synuclein caused a 15% reduction of TH+ neurons in the lesioned SNc of control mice (5,086 ± 292.8 cells). This loss was significantly more pronounced (4,231 ± 208.2 cells) in Bcl11a cko mice (Figure 7D). The possibility that the decrease in neuronal counts after rAAV injection may reflect a downregulation of TH expression rather than actual cell degeneration was ruled out by counting the total number of Nissl-stained neurons. This number declined by 12% (from 9,185 ± 141.4 to 8,070 ± 525.6) and 27% (to 6,741 ± 326.1) in the lesioned SNc of control and Bcl11a cko mice, respectively (Figure 7E). Hyperphosphorylation is a consequence of intraneuronal α-synuclein accumulation and a marker of pathological changes in α-synuclein conformation and aggregation (Fujikawa et al., 2002; Migdaelska-Richards et al., 2017). Midbrain sections from control and Bcl11a cko mice with rAAV-induced α-synuclein overexpression were immunostained with an antibody recognizing α-synuclein phosphorylated at Ser129 (p129-α-synuclein). Staining for p129-α-synuclein was virtually absent within SNc neurons on the intact side of the SNc (data not shown), whereas robust immunoreactivity characterized neurons in the injected SNc, consistent with an accumulation of pathological α-synuclein. The density of labeled neurons was decreased in the right SNc of Bcl11a cko mice as compared to control mice, likely reflecting the more pronounced nigral cell loss seen in Bcl11a-deficient animals (Figure 7F). Taken together, these findings reveal that vulnerability to α-synuclein toxicity is markedly enhanced in the absence of BCL11A and support the notion that BCL11A expression is associated with the transcription of genetic information involved in neuroprotective pathways.

The Bcl11a-expressing SNc-mDA neuronal subpopulation is highly vulnerable to toxic insults

Additional experiments using the same paradigm of rAAV-induced α-synuclein overexpression were carried out in Bcl11a cko lacZ (n = 4) and Bcl11a-lacZ (genotype: Bcl11a flox/lacZ ) control (n = 5) mice. Like the results in Bcl11a cko mice without the lacZ allele, α-synuclein toxicity caused a more severe loss of TH-positive neurons in the SNc of Bcl11a cko mice (from 5,954 ± 71.1 to 3,349 ± 193.3 cells) than that of Bcl11a-lacZ controls (from 5,954 to 5,041 ± 81.6 cells) (Figure 7G). In these experiments, confocal stereological counts were also able to distinguish the effects of α-synuclein overexpression on Bcl11a-mDA neurons in the SNc (TH+ and β-gal+) versus SNc-mDA neurons devoid of Bcl11a (TH+ and β-gal-). In Bcl11a-lacZ mice, 30% of β-gal+ neurons (from 1,899 ± 70.8 to 1,332 ± 93.7 cells) degenerated because of α-synuclein overexpression, whereas no statistically significant changes were observed in the number of β-gal− mDA cells between the lesioned and intact SNc (from 4,055 ± 118.8 to 3,709 ± 83.5 cells) (Figure 7H). These data indicate that Bcl11a expression characterizes a subpopulation of SNc-mDA neurons highly susceptible to α-synuclein-induced damage.

The number of β-gal+ and β-gal− neurons was then counted in the SNc of Bcl11a cko lacZ animals. Results showed more dramatic toxic effects because α-synuclein toxicity killed almost 65% (from 1,899 to 664 ± 117.0 cells) and 34% (from 4,055 to 2,685 ± 91.4 cells) of β-gal+ and β-gal− cells, respectively (Figure 7I). Thus, in the absence of BCL11A expression, Bcl11a-mDA neurons became even more vulnerable to neurodegeneration, consistent with the protective role of BCL11A-mediated transcription described in the previous section. Interestingly, in Bcl11a cko lacZ mice, neuronal cell death also affected β-gal− mDA neurons. This latter finding suggests that inactivation of Bcl11a may have cell non-autonomous effects resulting in widespread deleterious consequences for nigral tissue integrity.

To further explore the vulnerability of Bcl11a-expressing SNc-mDA neurons to toxic insults, we analyzed scRNA-seq data from hiPSC-derived mDA neurons that were treated with rotenone to induce oxidative stress (Fernandes et al., 2020). Rotenone treatment significantly reduced the proportion of SNc-like mDA neurons (DAN1; Figure 2A). Further investigation highlighted a subpopulation of DAN1 cells sensitive to rotenone. Because
this subpopulation was absent from the rotenone-treated culture, we inferred those sensitive cells by mapping transcriptomes from the treated DA11 cells to the untreated culture population. Cells with no pair in the untreated population were considered sensitive to rotenone. Interestingly, these rotenone-sensitive DA11 cells had a distinct transcriptomic profile, with BCL11A more likely to be detected within single cells in this group than the rest of the non-sensitive DA11 cells (BCL11A-negative cells: 407 depleted, 495 survived; BCL11A-positive cells: 83 depleted, 63 survived; Fisher’s exact test for count data, \( p = 0.0002017 \) for days, and \( p = 0.0076354 \) for interdependence between days and genotypes). Error bars indicate mean ± SEM.

**DISCUSSION**

mDA neurons are diverse in their gene expression, their anatomical location, their projection targets, and their electrophysiological and functional properties (Farassat et al., 2019; Morales and Margolis, 2017; Poulin et al., 2020; Roeper, 2013). How these different features of mDA diversity are related is not yet clear but is the subject of intense research. For example, a number of recent studies have shown that subsets of mDA neurons sharing expression of specific markers have also specific projection targets in the forebrain (Bimpisidis et al., 2019; Heymann et al., 2020; Khan et al., 2017; Kramer et al., 2018; Poulin et al., 2018). However, how and whether such subsets are already defined during development remain unclear.

Here, we show that the expression of the transcription factor BCL11A defines subpopulations of mDA neurons starting from embryogenesis and throughout adulthood in the mouse and in iPSC-derived human mDA neurons. BCL11A is expressed in many types of neurons in almost every region of the CNS (Allen Brain Atlas), but its function has been studied only in a few regions so far (Simon et al., 2020). In the cortex, BCL11A is important for the specification of cortical neurons that project to subcerebral areas (Cánoas et al., 2015; Woodworth et al., 2016), and it controls the acquisition of sensory area identity and the establishment of sensory input fields (Greig et al., 2016). Moreover, BCL11A regulates the migration of cortical projecting neurons (Wiegrefe et al., 2015). In the dorsal spinal cord, BCL11A is required for neuronal morphogenesis and sensory
Figure 7. a-Synuclein toxicity is enhanced in the absence of BCL11A
(A) Mice received a unilateral intraparenchymal injection of rAAVs carrying the DNA for human a-synuclein (hα-syn) into the SNc.
(B–F) Control (n = 5) and Bcl11a cko (n = 5) mice were sacrificed 8 weeks after treatment with hα-syn-AAVs. (B) Midbrain coronal sections comprising the SNc (delineated with dotted lines at low magnification) were immunostained with anti-human a-synuclein antibody to detect expression of the transduced protein in control and Bcl11a cko mice. Right panels: higher magnification of the boxed areas. (C) Midbrain sections were immunostained for TH (brown staining) and counterstained with cresyl violet (purple). Left panels: lesioned (AAV-injected) sides. Right panels: contralateral intact sides. (D and E) TH-immunoreactive (D) and Nissl-stained (E) neurons were counted stereologically in the intact (left) and lesioned (right) SNc of control (n = 5) and Bcl11a cko (n = 5) mice. Intact side values from control and cko animals were pooled (dark gray bars). Light-gray and empty bars show average values from the lesioned side of control (ctr) and cko mice, respectively. (F) Midbrain sections were immunostained for p129 a-synuclein. Representative images show labeled mDA cell bodies in the SNc; the square boxes encompass areas of the SNc that are shown at higher magnification (insets).

(G–I) Bcl11a-lacZ control (n = 5) and Bcl11a cko-lacZ (n = 4) mice were sacrificed 8 weeks after treatment with hα-syn-AAVs. Midbrain sections from Bcl11a-lacZ control (n = 5) and Bcl11a cko-lacZ (n = 4) mice were double stained with anti-TH and anti-β-gal antibodies. (G) Total neurons (β-gal-negative and β-gal-positive TH cells) were counted in the SNc of Bcl11a-lacZ and Bcl11a cko-lacZ mice using confocal stereology. (H) The number of TH/β-gal-positive and TH/β-gal-negative

(legend continued on next page)
circuit formation (John et al., 2012). Whether it functions purely as a transcriptional repressor or also as part of the chromatin remodeling complex in these neurons remains to be investigated (see Introduction). In mDA neurons, the inactivation of Bcl11a results in a rostral-to-caudal shift in the position of Bcl11a-mDA neurons in the VTA and a medial-to-lateral shift in the rostral SNc, suggesting that BCL11A might play a role in regulating the expression of factors that control the migration of mDA neurons. In the cerebral cortex, BCL11A regulates cortical neuron migration by controlling the expression of Semaphorin 3c (Wiegreffe et al., 2015), a cell adhesion molecule that is not expressed in mDA neurons (Kolk et al., 2009). An analysis of the scRNA-seq data for BCL11A-positive and BCL11A-negative cells in the hiPSC-derived DAn1 and DAn4 population showed that in BCL11A-positive cells, genes encoding components of the cytoskeleton or cytoskeleton-associated proteins known to be involved in the regulation of neuronal migration are differentially expressed (e.g., doublecortin-like kinase, neuronal navigator 1, tubulin beta 2 chain in DAn1; Rac1, microtubule associated protein 1B and tubulin beta 2 chain in DAn4) (Breuss et al., 2017; Deuel et al., 2006; Kawauchi, 2015; Koizumi et al., 2006; Sánchez-Huertas et al., 2020; Villarroel-Campos and González-Billault, 2014). Thus, inactivation of Bcl11a in mDA neurons could result in changes in cytoskeleton dynamics that contribute to altered neuronal migration and positioning of mDA neurons in Bcl11a cko mice.

In humans, pathogenic variants of BCL11A (mostly de novo mutations) result in neurodevelopmental disorders that have recently been classified as BCL11A-related intellectual disabilities (Peron et al., 2019). This syndrome is characterized by general developmental delay, microcephaly, speech delay, and behavioral problems, as well as several non-CNS-related phenotypes. In some affected individuals, seizures or autism spectrum disorder have been reported. Given the widespread expression of BCL11A in the brain, it is unclear if any of these neurological symptoms in patients are associated with functional deficits in the DA system. Moreover, there are no reports of neurodegenerative phenotypes, but long-term observations of patients or case studies in adult or aged patients are not available, due to the limited number of cases reported and the bias toward diagnosis in childhood (Peron et al., 2019).

DA subcircuits may encode a precise behavioral output by targeting a set of substructures within the DA projection targets, and a few studies have shown that specific subcircuits within the mDA system regulate very specific aspects of behavior (Engelhard et al., 2019; Heymann et al., 2020; Menegas et al., 2018). Whether such behavioral modules are consistent with genetically determined populations is just starting to be examined (Heymann et al., 2020). Bcl11a-mDA neurons of the VTA project to the medial and ventral shell of the NAc and the OT, whereas Bcl11a-mDA neurons of the SN show a highly selective innervation of the ventral TS and the caudal DMS. This specific innervation pattern suggests that this genetically defined subpopulation may modulate a specific subset of dopamine-influenced behaviors. Indeed, inactivation of Bcl11a in mDA neurons results in a specific behavioral impairment in skilled motor learning, whereas spontaneous motor behavior or coordination is not affected. Because Bcl11a is specifically inactivated in mDA neurons in our conditional knockout mouse model, this behavioral phenotype is most likely caused by functional changes in mDA neurons rather than by deficits in other midbrain neurons, cerebellum, or motor cortex (Hiksaka et al., 2002; Li et al., 2017). Potential functional changes upon Bcl11a inactivation in mDA neurons, such as altered neuronal activity and/or a decrease in dopamine release, are apparently severe enough to elicit a behavioral phenotype. It will have to be explored in the future how Bcl11a inactivation alters Bcl11a-mDA neurons at a functional level. Such studies are complicated by the fact that only relatively small areas are densely innervated by Bcl11a-mDA neurons and that Bcl11a-mDA neurons constitute only a subset of mDA neurons. To date, BCL11A has not been directly linked to the regulation of neuronal activity. However, the fact that haploinsufficiency for BCL11A in humans leads to intellectual disability and BCL11A is a risk gene for autism spectrum disorder (as described above) indicate that BCL11A may influence neuronal activity not only by affecting brain development but also by directly regulating the expression of genes important for neuronal function in mature neurons (Peron et al., 2019; Satterstrom et al., 2020). Another line of evidence supporting a function of Bcl11 genes in regulating neuronal activity comes from studies of the closely related Bcl11b in hippocampal neurons. Inactivation of Bcl11b in the adult brain leads to changes in hippocampal synapse formation and maintenance as well as electrophysiological deficits (e.g., massively impaired LTP) (De Bruyckere et al., 2018; Simon et al., 2016).

Dopamine release in the dorsal striatum appears to be essential for learning skilled motor behavior. Partial ablation of mDA projections using 6-hydroxydopamine (6-OHDA) in rats does not affect spontaneous motor behavior in the open field or on balance beam, but it strongly interferes with the ability of the animals to improve their performance on the rotarod (Ogura et al., 2005). Recent evidence suggests that the precise timing of dopamine signals influences the performance of highly skilled motor behavior in an immediate and a long-term manner (Bova et al., 2020). It has been proposed that the plasticity in striatal medium spiny neurons underlying initial skill learning during goal-directed actions requires dopamine release in the DMS, whereas dopamine release in the DLS is important for optimal skill learning (Durieux et al., 2012; Graybiel, 2008). Based on this model and our observation that Bcl11a-mDA neurons in the SNc project to the DMS but not the DLS, the deficit in motor learning could have its origin in the inability of the mice to initiate the learning of skilled motor behavior. Interestingly, the ablation of Aldh1a1-expressing mDA neurons leads to an impairment in motor skill learning on the rotarod that is similar to the one observed in Bcl11a cko mice even though Aldh1a1-expressing neurons project almost exclusively to the DLS (Wu et al., 2019). Thus, it is
possible that altered dopamine release either in DMS and DLS and thus defects in both phases of learning lead to a similar overall defect in the acquisition of skill learning.

The mDA neurons in the ventral tier of the SNc have been characterized as being most vulnerable to neurodegeneration in PD as well as in animal models of the disease (Surmeier et al., 2017). Based on some PD models in rodents, the most vulnerable population appears to coincide with ALDH1A1-expressing SNC-mDA neurons (Poulin et al., 2014). On the other hand, ALDH1A1-negative SNC-mDA neurons are more vulnerable to overexpression of α-synuclein with a disease-relevant mutation (α-synuclein A53T) in a transgenic mouse model (Liu et al., 2014). Here, we demonstrate that Bcl11a-mDA neurons in the SNC, which are mostly located in the dorsal tier and are largely ALDH1A1 negative, are highly susceptible to neurodegeneration upon α-synuclein overexpression (mouse) or rotenone exposure (human). Thus, our data suggest that there are also differences in the vulnerability of mDA neurons within the dorsal tier of the SNC and that BCL11A serves as a marker for the more vulnerable mDA neuronal population within the dorsal tier. In the mouse brain, these Bcl11a-mDA neurons project to the caudal DMS, which corresponds roughly to the caudate nucleus in the human brain (Burton et al., 2015). Some of the early symptoms associated with PD are assumed to be based on functional deficits of the caudate nucleus (rapid eye movement sleep behavior disorder and gait problems), and a recent study demonstrated a significant DA de-innervation of the caudate nucleus in about one-half of the examined patients in the early stage of PD (Pasquini et al., 2019), suggesting that caudate-projecting mDA neurons may also form a vulnerable population in humans. BCL11A is expressed in hiPSC-derived mDA neurons (our data) and in human mDA neurons (La Manno et al., 2016), but further studies will be necessary to evaluate which subpopulations express this transcription factor in the human DA system and whether the reduced DA innervation of the caudate nucleus in early PD may be associated with a specific loss of BCL11A-expressing mDA neurons in patients.

Although Bcl11a-mDA SNC neurons are more susceptible to α-synuclein-induced degeneration, BCL11A also acts as a neuroprotective factor in this population because the loss of Bcl11a-mDA neurons is significantly more severe in Bcl11a ckolacZ mice than in Bcl11a-lacZ mice. BCL11A could be modulating cell survival directly because it has been shown to regulate expression of the anti-apoptotic factor BCL2 in early B lymphocytes and inactivation of Bcl11a in cortical projection neurons results in increased cell death (Wiegrefe et al., 2015; Yu et al., 2012). An analysis of scRNA-seq data of BCL11A-positive and BCL11A-negative cells in the DA1 population shows that several genes or pathways that have previously been implicated in neurodegenerative processes are differentially expressed in BCL11A-positive neurons. These include genes encoding for microtubule-associated proteins (described above: doublecortin-like kinase, neuronal navigator 1, and tubulin beta 2 chain). By regulating the expression of these genes, BCL11A could affect the balance between stable and dynamic microtubules. Because perturbances in microtubule dynamics might contribute to neurodegenerative processes, this could be one potential pathway regulated by BCL11A contributing to its role as a neuroprotective factor (Calogero et al., 2019; Dubey et al., 2015). In addition, we find that NSC1 (neuronal Ca2+ sensor 1) transcripts are upregulated in the BCL11A-expressing human DA11 population. NSC1 acts as a neuroprotective factor potentially by shielding SNc-mDA neurons from Ca2+ overload, which arises from the particular activity patterns (pacemaker activity) and low Ca2+ buffering capacities of SNc-mDA neurons. Inactivation of NSC1 in mice aggravates neurodegeneration in the SNc, and NSC1 levels are reduced in iPSC-derived mDA neurons from patients with familiar PD (Benkert et al., 2019; Surmeier et al., 2017). If BCL11A positively regulates NSC1 expression, a loss of BCL11A could lead to reduced NSC1 levels, which could contribute to increased susceptibility of these neurons to neurodegeneration. Future studies will have to investigate whether these mechanisms underlie the function of BCL11A in neurodegeneration and neuroprotection in both mouse and human mDA neurons.

CONCLUSIONS

Our data demonstrate that the expression of Bcl11a defines subpopulations of mDA neurons in the SNC and VTA that form a highly specific subcircuit within the DA system. We show in the mouse DA system and in hiPSC-derived mDA neurons that the Bcl11a-expressing mDA neurons in the SNC show distinct susceptibility to toxic insults. The loss of BCL11A function in these neurons interferes with their functional integrity and their resilience to neurodegeneration. It will be of great interest to investigate whether the subcircuits formed by Bcl11a-expressing mDA neurons comprise a specific functional module in the DA system and to understand the gene regulatory networks downstream of BCL11A that affect the (patho)physiology of this subpopulation of mDA neurons.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109697.
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AUTHOR CONTRIBUTIONS

M.T., data curation, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing; A.U., data curation, formal analysis, validation, investigation, visualization, methodology, writing—review and editing; K.U.S.I., investigation, visualization, methodology, writing—review and editing; N.P., data analysis—single-cell transcriptomics, visualization—single-cell transcriptomics, methodology—single-cell transcriptomics, writing—review and editing; G.O.B., investigation, visualization, methodology, writing—review and editing; E.O., B.B., A.M., and A.W., investigation, writing—review and editing; S. Britsch, P.L., W.T.K., and K.M.J.v. Loo, resources, writing—review and editing; E.M., data curation, resources, writing—review and editing; S. Baader, data curation, formal analysis, validation, resources, methodology, writing—review and editing; D.A.D.M., data curation, formal analysis, validation, resources, writing—review and editing; S. Blaess, conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, methodology, project administration, writing—original draft, writing—review and editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


### KEY RESOURCES TABLE

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**Bacterial and virus strains**

- **AAV1/2-EF1α-DIO-ChR2-eYFP** UKB Viral Core Facility, Bonn, DE N/A
- **pAAV-EF1α-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA** Addgene, Watertown, MA, USA 20297-AAV1
- **AAV1/2-EF1α-pTRE-FLEX-ChETA-eYFP** UKB Viral Core Facility, Bonn, DE N/A
- **AAV2/6-hSYN1-hα-synuclein-WPRE** Sirion Biotech, Martinsried, Germany Musgrove et al., 2019

**Critical commercial assays**

- **RNAScope® Fluorescent Multiplex Detection Reagents** Advanced Cell Diagnostic 323110
- **Mm-Bcl11a-C3 RNA probe** Advanced Cell Diagnostic N/A

**Deposited data**

- **Imaging and quantitative data** Mendeley Data Mendeley Data: https://doi.org/10.17632/4h9265nnrf.1
- **scRNaseq data** Fernandes et al., 2020 ArrayExpress: E-MTAB-9154

**Experimental models: Cell lines**

- **KOLF-2 iPSC (human)** HipSci RRID:CVCL_9S58

**Experimental models: Organisms/strains**

- **A82D (TITL-GFP)** The Jackson Laboratory RRID: MGI: J:219930
- **Bcl11aCreER** Dr. Walid Khaled, University of Cambridge Pensa et al., 2021
- **Bcl11aflx** Dr. Pentao Liu, The University of Hong Kong; Prof. Stefan Britsch, University of Ulm; RRID: J:194616
- **Bcl11aLacz** Dr. Pentao Liu, The University of Hong Kong RRID: J:219746
- **DATRES-Cre** The Jackson Laboratory RRID: MGI: J:114466
- **DatTA** The Jackson Laboratory RRID: MGI: J:218892

**Oligonucleotides**

- **Primers for A82D, see Table S3** The Jackson Laboratory N/A
- **Primers for A9, see Table S3** The Jackson Laboratory N/A
- **Primers for Bcl11a-flx, see Table S3** This paper N/A
- **Primers for Cre, see Table S3** The Jackson Laboratory N/A

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the Lead Contact, Sandra Blaess (sblaess@uni-bonn.de).

Materials availability
This study did not generate new unique reagents.

Data and code availability

- The accession number for the scRNA sequencing is ArrayExpress: E-MTAB-9154, as reported in Fernandes et al. (2020). Imaging and quantitative data have been deposited at Mendeley and are publicly available as of the date of publication. DOIs are listed in the Key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Bcl11a*^lacZ^ mice (Dias et al., 2016) were kindly provided by Pengtao Liu, School of Biomedical Sciences, The University of Hong Kong, China. *Bcl11a*^floxed^ mice (Wiegrefe et al., 2015) were kindly provided by Pengtao Liu, School of Biomedical Sciences, The University of Hong Kong, China; and Neal Copeland, Institute for Academic Medicine, Houston Methodist and obtained from Stefan Britsch, University of Ulm. *Bcl11a*^CreER^ mice (Pensa et al., 2021) were kindly provided by Walid Khaled, Department of Pharmacology, University of Cambridge. *Bcl11a* cko mice were generated by crossing *DatIRES-Cre* mice (Bäckman et al., 2006) with *Bcl11a*^floxed^ mice (Genotype: *DatIRES-Cre*^/-^, *Bcl11a*^floxed^). In a subset of *Bcl11a* cko mice, the *Bcl11a*^lacZ^ null allele was introduced by crossing *DatIRES-Cre* mice with *Bcl11a*^lacZ^ mice (Genotype: *DatIRES-Cre*^/-^, *Bcl11a*^lacZ^). Intersectional fate mapping experiments were performed by crossing...
**Bcl11aCreER** mice with the DatTA (tetracycline trans-activator driven by the Dat promoter) mouse line (Chen et al., 2015) and the intersectional reporter mouse line Ai82D (Ai82(TITL-GFP)-D (Madsen et al., 2015) (Genotype: **Bcl11aCreER/+, DatTA/- Ai82D(TITL-GFP+)). For viral tracings, **Bcl11aCreER/+ or Bcl11aCreER/+, DatTA/+** (intersectional) were used. Mice were kept on a mixed C57BL6/CD1 background. Developmental stages are indicated in the Results and Figure legends or in the methods section (for behavioral analysis and α-synuclein overexpression experiments). Mice were group housed in a controlled environment, with 12 hr light/night cycles and ad libitum availability of food and water. Day of vaginal plug was recorded as E0.5. All experiments were performed in strict accordance with the regulations for the welfare of animals issued by the Federal Government of Germany, European Union legislation and the regulations of the University of Bonn. The protocol was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Permit Number: 84-02.04.2014.A410, 84-02.04.2016.A238 and 84-12.04.2015.A550). Both female and male mice were used for multiplex RNA in situ analysis, immunostaining, viral tracing experiments and α-synuclein overexpression. Male mice were used for behavioral experiments. For comparison of **Bcl11a cko and control mice, littermates were used (from several litters).**

**Tamoxifen administration**
Tamoxifen was administered by oral gavage to pregnant dams at E15.5 (0.05 ml/10 g body weight) or to adult mice (0.075 ml/10 g body weight) to label **Bcl11a**-expressing neurons. Tamoxifen (Sigma Aldrich) was prepared as a 20 mg/mL solution in corn oil (Sigma Aldrich).

**Tissue processing**
Pregnant females were sacrificed by cervical dislocation. Embryos were transferred into ice cold PBS, decapitated and dissected. P0 pups were decapitated, and their brain dissected in ice cold PBS. Heads (E12.5 – E15.5) or brains (P0) were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Adult mice were anesthetized with an intraperitoneal injection of Ketanest/Rampun or pentobarbital and subsequently perfused transcardially with phosphate buffered saline (PBS), followed by 4% PFA. The tissue was cryopreserved in OCT Tissue Tek (Sakura). Embryonic and P0 tissue was cryosectioned at 14 μm thickness and collected on glass slides, adult brains were cryosectioned at 40 μm thickness and free-floating sections were collected in anti-freeze solution.

For immunofluorescent staining, sections were re-fixed in 4% PFA for 10 min at room temperature (RT) and incubated in 10% NDS in PBS plus 0.2% Triton X-100 (Sigma-Aldrich) (0.2% PBT, used for embryonic and P0 tissue) or in 10% NDS in 0.5% PBT (adult tissue) for 1 hr at RT. Sections were incubated with primary antibody overnight at 4°C in 3% NDS in 0.2% PBT (embryonic and P0 tissue) or in 3% NDS in 0.3% PBT (adult tissue). For staining with the guinea pig anti-BCL11A antibody and in some cases for rabbit anti-TH antibody sections were incubated in the primary antibody overnight for 72 hr at RT (guinea pig anti-BCL11A) or at 4°C (rabbit anti-TH antibody). Sections were washed 3 times for 5-10 min in 0.2% PBT (embryonic and P0 tissue) or in 0.3% PBT (adult tissue) and incubated for 2 hr at RT in secondary antibody in 3% NDS in 0.2% PBT (embryonic and P0 tissue) or in 3% NDS in 0.3% PBT (adult tissue). Sections were washed 3 times for 5-10 min in 0.2% PBT (embryonic and P0 tissue) or in 0.3% PBT (adult tissue) and mounted with Aqua Polymount (Polysciences Inc.). For the detection of BCL11A, biotinylated donkey anti-guinea pig antibody followed by Cy3-Streptavidin was used.

Processing of tissue from mice overexpressing α-synuclein involved post-fixation with 4% PFA for 24 hr followed by cryopreservation in 30% sucrose solution. Sections were cut at 35 μm thickness using a freezing microtome. Staining of these sections followed previously described protocols (Helwig et al., 2016). A list of primary and secondary antibodies is provided in Key resources table. The following primary antibody dilutions were used: 1:500 to 1:1000 for rabbit anti-TH, 1:500 for mouse anti-TH and rabbit anti-SOX6, 1:1000 for rabbit anti-ALDH1A1 and rabbit anti-RFP, 1:2000 for goat anti-β-gal and rat anti-GFP, 1:2500 for guinea pig anti-BCL11A, 1:5000 for rabbit anti-Calbindin and 1:20,000 for mouse anti-human α-synuclein. Biotinylated donkey anti-guinea pig antibody was used at a 1:200 dilution and Cy3-streptavidin at a 1:1000 dilution. Cy3-conjugated anti-goat Cy3 and all Alexa fluorophore-conjugated secondary antibodies were used at a 1:500 dilution.

**Multiplex fluorescent in situ hybridization**
Multiplex fluorescent RNA in situ hybridization on frozen sections from P0 and adult mice was performed using RNAscope Fluorescent Multiplex Detection Reagents (323110, ACDBio, Newark, CA, USA) according to the instructions provided by the manufacturer for frozen tissue (User Manual: 323100-USM). Hybridized probe was detected with TSA Plus Cyanine 3 (NEL760001KT, Perkin Elmer, Waltham, MA, USA). The probe for **Bcl11a** was designed by ACDBio (Cat No. 563701-C3). Sections were counterstained with TH and Hoechst, then mounted with Aqua Polymount (Polysciences Inc., Warrington, PA, USA).

**Stereotactic viral vector injection**

**Viral tracing**
**Bcl11aCreER** and **Bcl11aCreER**, DatTA mice (4-20 weeks old) were anesthetized with Fentanyl/Midazolam/Medetomidin and placed into a stereotaxic apparatus. 1 μL of virus solution with a titer of Titer ≥ 1 × 1013 vg/mL (AAV1/2-EF1α-pTRE-FLEX-ChETA-eYFP or AAV1/2-EF1α-DIO-ChR2-eYFP (both from UKB viral core facility, Bonn, DE) or AAV1/2-EF1α-DIO-ChR2-mCherry (Addgene, Water-town, MA, USA)) was injected unilaterally into the SNc (from Bregma: anteroposterior (AP), −2.3 mm; mediolateral (ML), −1.1 mm; dorsoventral (DV), −4.1 mm) or into the VTA (from Bregma: AP, −3.44 mm; ML, −0.48 mm; DV, −4.4 mm). A 34 g beveled needle (WPI)
and a microinjection pump (WPI) were used to control the injection speed (100 nl/min). After the injection, the syringe was kept in place for 3 min and slowly retracted over 1 min. 8 days after viral injections Tamoxifen was administered to the mice by oral gavage (0.075 ml/10 body weight) for 3 consecutive days. Two weeks after the last Tamoxifen administration, mice were perfused.

**Alpha-Synuclein overexpression**

Recombinant adeno-associated viral particles (serotype 2 genome and serotype 6 capsid) were used to express human α-synuclein in the mouse substantia nigra. Gene expression was controlled by the human Synapsin 1 promoter and enhanced using a woodchuck hepatitis virus post-transcriptional regulatory element and a polyA signal downstream to the α-synuclein sequence. AAV-vector production, purification, concentration, and titration were performed by Sirion Biotech (Martinsried, Germany). Mice (5-8 months for comparison between Bcl11a cko<sup>blox</sup> and control mice; 5-16 months for comparison between Bcl11a cko<sup>lacZ</sup> and Bcl11a-lacZ mice) were treated with a single 1.5 μl injection of 4.0×10<sup>12</sup> genome copies/ml using a stereotactic frame with a mouse adaptor (Stoelting, Wood Dale, IL, USA) under isoflurane anesthesia. Stereotaxic coordinates were 2.3 mm posterior and 1.1 mm lateral to bregma; injection depth was 4.1 mm relative to dura mater. The injection was made at a rate of 0.4 μl/min using a Hamilton syringe fitted to a glass capillary. The capillary was left in position for an additional 5 mins before being retracted. A list of AAVs is provided in Key resources table.

**Image acquisition**

Images of fluorescently stained images were acquired at an inverted Zeiss AxioObserver Z1 equipped with structured illumination (ApoTome) and a Zeiss AxioCam MRm (Carl Zeiss, Oberkochen, DE). At 10X (EC PlanN 10x/0.3, Carl Zeiss, Oberkochen, DE) magnification, tile images were acquired with conventional epifluorescence. At 20X (EC PlanN 20x/0.5, Carl Zeiss, Oberkochen, DE), 40X (Pln Apo 40x/1.3 Oil, Carl Zeiss, Oberkochen, DE) and 63X (Pln Apo 63x/1.4 Oil, Carl Zeiss, Oberkochen, DE) magnifications, structured illumination was used to acquire tile images and z stacks. Some of the images taken with the 20X objective and all the images taken with the 40X and 63X objective are maximum intensity projections of z stacks. Tile images were stitched with Zen blue software (Zeiss, 2012).

Brightfield images were visualized with a Zeiss Axioscope.A1 microscope, collected using AxioCam 503 Color and processed with Zen blue software (Zen lite, 2019). In situ hybridized sections at adult stages were imaged at an inverted Zeiss AxioObserver equipped with a CSU-W1 Confocal scanner unit (50 μm pinhole disk, Yokogawa, Tokyo, JP). At 40X (C-Apochromat, 40x/1.2 water, Zeiss) magnification, tile images and z stacks were acquired with laser lines 405 nm, 488 nm and 561 nm. Images taken with the 40X objective are maximum intensity projections of z stacks. Tile images were stitched with VisiView software (Visitron Systems, Puchheim, DE).

**Quantification of cell numbers**

**TH<sup>+</sup> β-gal<sup>+</sup> neurons and additional subset markers**

The percentage of Bcl11a-expressing mDA neurons in SN or VTA at P0 and P30 was determined by quantifying TH<sup>+</sup> β-gal<sup>+</sup> neurons at four (P0) or three (P30) rostrocaudal midbrain levels (Franklin and Paxinos, 2007). TH<sup>+</sup> β-gal<sup>+</sup> neurons in CLi and RRF in the adult brain were analyzed separately from neurons in the SN or VTA. The number of TH<sup>+</sup> β-gal<sup>+</sup> neurons was counted unilaterally in the SN, VTA, RRF and CLi and normalized for the total number of mDA neurons per region separately. The analysis was performed on n = 5 Bcl11a-lacZ control mice and n = 5 Bcl11a cko<sup>lacZ</sup> mice at P0, and on n = 6 Bcl11a-lacZ (control) mice and n = 6 Bcl11a cko<sup>lacZ</sup> mice at P30. The percentage of Bcl11a-expressing mDA neurons co-expressing additional subset markers (SOX6 and CALB1 at P0; CALB1 and ALDH1A1 at P30) in SN or VTA was determined by quantifying TH<sup>+</sup> β-gal<sup>+</sup> neurons at four rostrocaudal midbrain levels (Franklin and Paxinos, 2007). The number of TH<sup>+</sup> β-gal<sup>+</sup> neurons co-expressing the respective subset marker in these regions was counted unilaterally and the numbers were normalized for the total number of TH<sup>+</sup> β-gal<sup>+</sup> mDA neurons in each region (SN or VTA). This analysis was performed for n = 5 Bcl11a-lacZ mice and n = 5 Bcl11a cko<sup>lacZ</sup> mice at P0 and for n = 3 Bcl11a-lacZ mice and n = 3 Bcl11a cko<sup>lacZ</sup> mice at P30.

**Stereology**

Analyses were performed on SNc samples by an investigator blinded to the sample codes. Unbiased stereological estimates of the number of nigral neurons were obtained by counting under brightfield or confocal microscopy. Samplings were performed on every fifth section throughout the entire SNc. Delineations were made using a 4x objective, and counting was performed using a 63x Plan-Apo oil objective (Numerical aperture = 1.4). A guard zone thickness of 1 μm was set at the top and bottom of each section. Cells were counted using the optical fractionator technique (Stereo Investigator software version 9, MBF Biosciences, Williston, VTA, USA) using a motorized Olympus microscope (IX2 UCB) equipped with an Olympus disk spinning unit (DSU) and a light sensitive EM-CCD camera. Coefficient of error was calculated according to (Gundersen and Jensen, 1987); values < 0.10 were accepted.

**Analysis of fiber density**

**TH-expressing fibers**

40 μm sections of 3 to 6 rostrocaudal levels of the striatum were stained for TH and epifluorescence images were taken using and inverted Z1 Axioobserver microscope with 10X objective. The mean gray value of the TH<sup>+</sup> striatal projections was calculated in the dorsal striatum, OT and TS (divided into ventral and dorsal TS) with Fiji/ImageJ and normalized for background fluorescence (corpus...
EGFP-expressing fibers (intersectional genetic labeling)
40 μm sections of 5 rostrocaudal levels of the striatum were stained for TH and EFGP and epifluorescence images were taken using an inverted Z1 Axioobserver microscope with 10X objective. The mean gray value of the EGFP+ projections was calculated in the OT, NAc (divided into core and shell of the NAc), the rostral striatum (RS), DS (divided into medial and lateral DS) and TS (divided into ventral and dorsal TS) with Fiji/ImageJ and normalized for background fluorescence (cortex which is devoid of EGFP+ fibers). More specifically, EGFP+ fibers in the OT were analyzed at Bregma 1.42mm, 0.95mm, 0.62 mm and −0.15 mm. For the analysis of EGFP+ projections to the NAc core and shell and the RS, the mean gray value was calculated at Bregma 0.95mm and 1.42mm, respectively. EGFP+ fibers in the medial and lateral DS were analyzed at Bregma 0.62 mm and −0.15 mm, while those in the dorsal and ventral TS were analyzed at Bregma −0.58mm. The analysis was performed for 7 mice at P30.

EGFP-expressing fibers (stereotactic viral vector injections)
40 μm sections from one to three rostrocaudal levels of the striatum per animal were stained for TH and EFGP and epifluorescence images were taken using inverted Z1 Axioobserver microscope with 10X objective. The mean gray value of the EGFP+ projections was calculated in the OT (devided into medial and lateral OT) and NAc (divided into core and shell of the NAc) with Fiji/ImageJ. Subsequently, the background fluorescence (cortex which is devoid of EGFP+ fibers) was subtracted and the obtained value normalized for the average gray value of the analyzed areas to account for differences in recombination efficiencies between animals. This analysis was performed for 6 mice.

Behavioral tests
Male mice were kept ad libitum in groups of 2-3 animals in a 12-hour day/night rhythm on a normal diet. Animals ranging from two to four months of age were transferred to the examination room for experimental purposes and each one was housed in a separate cage. After a one-week acclimatization period in the examination room, mice performed the rotarod test and beam walking assay (both tests in one day) for five consecutive days, followed by a social recognition test (data not shown) and finally by the open-field test. All behavioral tests were performed with the experimenter being blinded to the genotype of the mice.

RotaRod
The animals were taken out of the cage and were acclimated to the rotarod apparatus (Ugo Basile, Gemonio, IT; Code no. 47600) in a 5-minute run on a rod rotating at a constant speed of 8 rpm. Afterward the animals went through three test runs per day, with a break of 30 min between each run. In each test run, the animals had to balance on the rotating rod for 5 min, whereby the torque increased from 4 to 40 rpm within 5 min. The duration that each mouse was able to stay on the rotating rod in each trial was recorded as the latency to fall. The three test runs per day were repeated on 5 consecutive days. During the test, the rod was kept clean and dry by wiping the mouse urine and feces off.

Beam Walking Assay
To examine fine motor skills independent of rotational movement (Luong et al., 2011), the beam walking assay (balanced beam test) was used (Carter et al., 1999). Animals had to balance on one platform over a 12 mm wide and 1 m long rod made of synthetic material to another platform that held a box with a food reward. Beams were placed 50 cm above the table. The time taken for the animals to cross the bar was measured. If animals did not reach the safety platform or took longer than 60 s to cross the beam, a maximum of 60 s was assigned. The test was run for a period of 5 consecutive days.

Open-field test
In the open-field test, the mice were placed in an open arena (30 × 30 × 30 cm). They were allowed to move freely for 5 minutes. The animals were recorded by Video (EthovisionXT, Noldus, Wageningen, NL) and the running distance, the time spent in the border area, the corners and the center of the cage as well as crossings from the border to the center area were measured. This test was initially run for a training period of 1 day, then after a 30-day rest period another test run was performed. This test was run twice with a 30-day rest period in between. Since both runs showed comparable results, the results were combined for the final analysis.

Single cell RNA-sequencing
Processing, QC and normalization
The single-cell dataset used with the same processing QC and cell type annotation as described in Fernandes et al. (2020).

BCL11A Expression Visualization
To show BCL11A single cell expression on the uniform manifold approximation and projection (UMAP), BCL11A transcript counts were normalized by their cell’s total detected transcripts, scaled to 10000 reads/cell and then log-normalized. For the barplots the BCL11A transcripts for every single-cell were transformed to counts per million. The error bars correspond to the variation observed within the cell type including dropouts.

BCL11A Dan1 transcriptomic signature and gene set enrichment analysis
To define genes that are related to BCL11A expression we split the Dan1 cells into two groups: BCL11A+ cells with at least 1 transcript and BCL11A- cells, with no detected transcripts. To identify differentially expressed genes we removed the BCL11A expression values from the transcriptome, since this was the main criterion of separation, and used scnnpy (Wolf et al., 2018) to perform the
wilcoxon rank sum test between the two groups. For gene set enrichment analysis, we used Metascape (Zhou et al., 2019) considering the first 100 upregulated differential expressed genes sorted by according to their statistical test p value.

**Neuronal vulnerability in BCL11A+ DAn1 neurons**

To assess how relevant BCL11A expression is to neuronal vulnerability we examined two events in every DAn1 neuron: a) whether it is annotated as sensitive to rotenone treatment as described in (Fernandes et al., 2020) and b) whether BCL11A is detectable. We examined the relation of those two events using the Fisher’s Exact test on the whole DAn1 population.

**STATISTICAL ANALYSIS**

Statistical analyses of cell numbers were performed with GraphPad Prism (9.0) software using unpaired t test and Welch’s t test when comparing two groups and one-way ANOVA followed by test for linear trend, one-way ANOVA followed by Tukey’s or Šidák’s multiple comparison test or Welch’s ANOVA followed by Dunnett’s T3 multiple comparison test when comparing multiple groups. Welch’s t test and Welch’s ANOVA were performed for groups of data that were sampled from Gaussian populations, but did not have the same standard deviation. Statistical analyses of behavioral experiments were done with R studio. Open field tests were evaluated by one-way analysis followed by Tukey’s post hoc test for multiple comparisons. Differences in the balanced beam test and rotarod were assessed by two-way ANOVA taking time and genotype as numerical and categorical variables. Samples size estimation was not performed. For comparison between controls and Bcl11a cko animals, animals were assigned to experimental groups according to genotypes. No data or subjects were excluded from the analysis, except for experiments involving stereotactical injections. In this case, subjects were excluded if expression of the protein after viral transduction was not detected in the targeted region (SNc or VTA). Statistical details are described in the Figure legends, the Results or in the STAR method section (for single cell RNA-sequencing). P values of less than 0.05 were considered statistically significant. Data are reported as mean values ± standard error of the mean (SEM).