DNMT1-dependent regulation of cortical interneuron function and survival

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Increased occurrence of age-associated disabilities and neurodegenerative diseases is the price we pay for the tremendous elevation in life expectancy in our modern society. Aging comes along with structural, neurochemical and physiological alterations in the brain that cause memory decline and cognitive impairments (Rozycka and Liguz-Lecznar, 2017). Numerous factors contribute to cognitive aging including hormonal, metabolic, and immune dysregulation, elevated oxidative stress and inflammation, changes in neurotransmission, and diminished neurotrophic support of neurons (Rozycka and Liguz-Lecznar, 2017). Thereby, different brain regions and neuronal cell types are distinctively affected by the process of aging. Apart from reduced excitability and plasticity, the decline in inhibitory function represents a prominent feature of aged brains (Zimmer-Bensch, 2019a). A selective vulnerability of inhibitory interneurons and GABAergic (gamma-aminobutyric acid) synapses is reported for diverse regions of the aged brain across different species. This is reflected by reduced numbers of inhibitory cortical interneuron subtypes, as well as by functional and structural changes of GABAergic synapses (Rozycka and Liguz-Lecznar, 2017; Zimmer-Bensch, 2019a).

The different types of GABA-expressing interneurons mediate local inhibition in the cerebral cortex as the seat of higher cognitive function, hence being key for cortical information processing (Zimmer-Bensch, 2019a). Due to their important role in cortical circuits, age-associated defects in the cortical GABAergic system represent an attractive hypothesis for the age-related cognitive decline and disorders (Rozycka and Liguz-Lecznar, 2017).

In line with the aforementioned age-associated structural alterations, changes in the expression of genes related to GABAergic transmission were reported frequently (Zimmer-Bensch, 2019a). Besides, an augmented expression of neuroprotection-related genes and the diminished expression of genes implicated in general synaptic function emerge as conserved features of mammalian brain aging (Zimmer-Bensch, 2019a). In agreement with this, transcriptome analysis of synaptosomes from aged murine cerebral cortices revealed altered expression of synaptic transmission-related genes (Rozycka and Liguz-Lecznar, 2017; Zimmer-Bensch, 2019a). In addition to protein-coding genes, differential expression of diverse long non-coding RNAs (lncRNAs) was detected between young and old synaptosomes. LncRNAs represent important epigenetic players, which in addition to transcriptional and post-transcriptional control in the nucleus can modulate translation in the cytoplasm through different mechanisms, and hence contribute to translational control at synapses (Zimmer-Bensch, 2019b).

Other epigenetic mechanisms of transcriptional control such as histone modifications and DNA methylation catalyzed by DNA methyltransferases (DNMTs) were further shown to be implicated in age-associated neuronal impairments (Zimmer-Bensch, 2019a). DNA methylation signatures have been described to be altered upon aging in human and mouse brains. However, apparent region-specific differences and the general challenge of correlating changes in methylation marks with the transcriptional output, as well as with physiological and biological responses, hamper general conclusions about functional implications (Zimmer-Bensch, 2019a).

The methylation of DNA, occurring mainly at cytosines, is a reversible and dynamic process, catalyzed by enzymes of the DNMT family, while active demethylation is achieved via oxidation by ten-eleven translocation proteins with subsequent iterative oxidation and base excision repair (Zimmer-Bensch, 2019a). Together, these mechanisms enable the dynamic reconfiguration of DNA methylation signatures, observed in the developing, adult and aged brain. In addition to DNMT-mediated DNA methylation, which is often associated with transcriptional silencing, DNMTs can act non-canonically through a crosstalk with histone modifications (Zimmer-Bensch, 2019a).

It is well accepted that DNA methylation, but also histone modifications and the expression of non-coding RNAs are responsive to external stimuli, such as changes in neuronal activity, stress or nerve injury (Zimmer-Bensch, 2019b). So, the observed age-related changes in the DNA methylation profiles could represent an adaptive response to the altered neuronal physiology like decreased synaptic activity, and the accompanied cellular changes. In that case, epigenetic mechanisms rather represent servants instead of being the masters.

In a previous study, we provided evidence that DNMT1 promotes the loss of cortical inhibitory interneurons seen in aged brains. Conditional deletion of *Dnmt1* in parvalbumin-positive interneurons attenuated their age-related reduction in the cerebral cortex, which was accompanied by reduced age-associated transcriptional changes in these knockout cells. In line with the critical functions of inhibitory interneurons in cortical information processing, we found that the conditional *Dnmt1*-deficient mice showed improved somatomotor performance (Hahn et al., 2020). However, when we compared the transcriptional profiles and DNA methylation signatures of the aged wild-type and *Dnmt1* knockout interneurons, the observed differences did not provide a logic explanation for a DNMT1-dependent regulation of cortical interneuron survival (Hahn et al., 2020). Concordant with the observation of the age-related decrease in DNMT1 activity, very few differentially methylated genes were identified between the aged genotypes (Hahn et al., 2020). In contrast to this, young *Dnmt1* deficient and control interneurons were distinguished by a prominent number of differentially expressed genes, very similar to the transcriptional changes which occurred upon aging in control mice (Hahn et al., 2020). For proper interpretation of these findings, a few aspects have to be considered.

An important point is the fact that transcriptome and methylome analyses at a discrete timepoint provide only a snapshot of the investigated stage, rather profiling the "consequences" than the "causes". To better understand how DNMT1 might affect cortical interneuron survival in the aged brain, analysis of younger stages has to be taken into consideration.

Analysis in young mice revealed that *Dnmt1* deletion in cortical interneurons lead to reduced DNA methylation and increased expression levels of endocytosis-related genes compared to equal-aged control samples (Pensold et al., 2020). This indicates that endocytosis-associated genes represent targets of repressive DNMT1-mediated DNA methylation. Functional analysis showed elevated endocytic rates and endocytosis-based vesicle recycling, which manifested in augmented GABAergic transmission by more efficient transmitter recycling (Pensold et al., 2020).

In contrast to this, numerous genes regulating neuronal excitability were down-regulated in *Dnmt1*-deficient cortical interneurons (without any respective changes in DNA methylation). This cannot be explained by the lack of canonical repressive DNMT1 function in the knockout samples and likely represented an adaptive response to the physiological effect of *Dnmt1* deletion: the elevation of GABAergic transmission. In addition to adaptive transcriptional changes, the *Dnmt1* deletion induced alterations in interneuron activity might have further triggered changes in the epigenetic make up, as neuronal activity was shown to alter the DNA methylation landscape (Guo et al., 2011). Hence, the *Dnmt1* deletion-mediated alterations in neuronal activity levels could secondarily lead to changes in DNA methylation signatures. Indeed, we found numerous genes with increased methylation levels in the *Dnmt1*-deficient samples (Pensold et al., 2020), which is in discordance with the well-known repressive DNA methylation function of DNMTs. Thus, when analyzing the biological meaning of an epigenetic writer such as DNMT1 by the use of knockout approaches as well as by overexpression studies, one has to take into consideration that direct effects, such as reduced/increased methylation of certain target genes, as well as adaptive changes in gene expression and DNA methylation profiles in response to the resulting altered cellular physiology, are triggered. This hampers the interpretation of the functional implications of the investigated proteins. Furthermore, both primary as well as secondary effects of *Dnmt1* deletion induced in young interneurons such as altered activity regulation might influence the interneuron survival upon aging.

Another functionally related group of genes we found significantly elevated in young *Dnmt1*-deficient interneurons, that presumably influences the long-term survival in aged mice, were genes related to the proteostasis network (Bayer et al., 2020). Proteostasis leads to the degradation and removal of defective proteins, which is of high importance for most of the neurons that do not regenerate. Diverse neurodegenerative diseases involve or rely on defects of the protein degradation machinery (Zimmer-Bensch, 2020). Hence, DNMT1 could indirectly regulate interneuron survival in aged mice by modulating the proteostasis network during life-time. By repressing genes related to proteostasis such as endosome and endo-lysosomal trafficking (Bayer et al., 2020; Hahn et al., 2020), DNMT1 could act as a "brake" in wild-type interneurons, reducing their proteostatic capacities. Upon aging and the accumulation of defective proteins this might render them sensitive and lead to higher interneuron cell death rates. As in *Dnmt1* knockout interneurons proteostasis-related gene expression

was found elevated (**Figure 1**), proteostatic processes might work more efficiently, which could cause their improved long-term survival. In line with that, we have shown that *Dnmt1* depletion ameliorates the mutant Huntingtin-induced cytotoxicity at least in part by acting on autophagy and aggresome formation (Bayer et al., 2020).

Huntington's disease (HD) is caused by a trinucleotide expansion mutation in the 50-coding region of the gene that encodes Huntingtin (HTT), manifesting in polyglutamine repeats. This causes the misfolding of the mutant HTT protein being highly prone to aggregate and to form intracellular inclusion bodies. Due to this, and the numerous functions and interactions mediated by the wild-type HTT protein, its mutation leads to impaired neurophysiology culminating in neurodegeneration of distinct neuronal subsets with different vulnerabilities (Zimmer-Bensch, 2020). In HD it is the population of striatal GABAergic projection neurons, the medium-sized spiny neurons, which is rendered most sensitive by the mutant HTT displaying a marked loss. Albeit less pronounced than in the striatum, the degeneration of particular cortical neurons was observed in HD patients, including mainly large pyramidal projection neurons of cortical layers V and VI (Zimmer-Bensch, 2020). However, the exact mechanisms of how DNMT1 and DNA methylation is involved in the mutant HTT cytotoxicity, remains to be elucidated. What it known so far is that changes in DNA methylation signatures have been reported in HD patients and transgenic mouse models. Such changes have been identified for genes related to neurodevelopmental processes, as well as for *ADORA2A*, encoding for the adenosine A2A receptor, a G-protein-coupled receptor, whose normally high expression in the basal ganglia is severely reduced in HD (Zimmer-Bensch, 2020). However, how this is mediated, and whether these altered DNA methylation marks represent direct consequences of mutant HTT, known to interact with epigenetic writers (Zimmer-Bensch, 2020), remains to be dissected in detail.

Another fact that complicates functional analysis of the physiological relevance of DNMTs and DNA methylation in age- and disease-related neurodegeneration, is that DNA methylation can have different transcriptional outcomes and biological consequences. In contrast to the conventional view of repressive DNA methylation by preventing the binding of transcription factors, DNA methylation profiles might even create new transcription factor binding motifs (Zhu et al., 2016). Besides, DNA methylation was shown to instruct alternative splicing and promoter choice (Lev Maor et al., 2015), increasing the functional spectrum enormously.

Apart from this, it is further accepted that there is extensive crosstalk between different epigenetic mechanisms (Symmank and Zimmer, 2017). While certain histone modifications favor DNA methylation, DNMTs can influence the establishment of histone marks directly by protein-interaction in enzyme complexes, or indirectly, by modulating the expression of related genes (Symmank and Zimmer, 2017). Non-coding RNAs, especially the lncRNAs, further intersect with DNA methylation in addition to histone modifications and miRNA pathways (Zimmer-Bensch, 2019b). Hence, an integrative genome-wide analysis has to be performed on a cell type-specific level, and at different stages in combination with comprehensive functional characterization, to better understand the epigenetic mechanisms that contribute to neuronal aging. To approach the underlying causes of the selective vulnerability of different neuronal subtypes, we need to discover the relation of the different epigenetic mechanisms to each other, as well as their responsiveness towards external influence, such as metabolic changes and alterations in neuronal activity. The enormous technological progress that is continuously achieved in the field of single cell sequencing, which can even be combined with electrophysiological characterization, might bring this challenging goal in feasible reach.

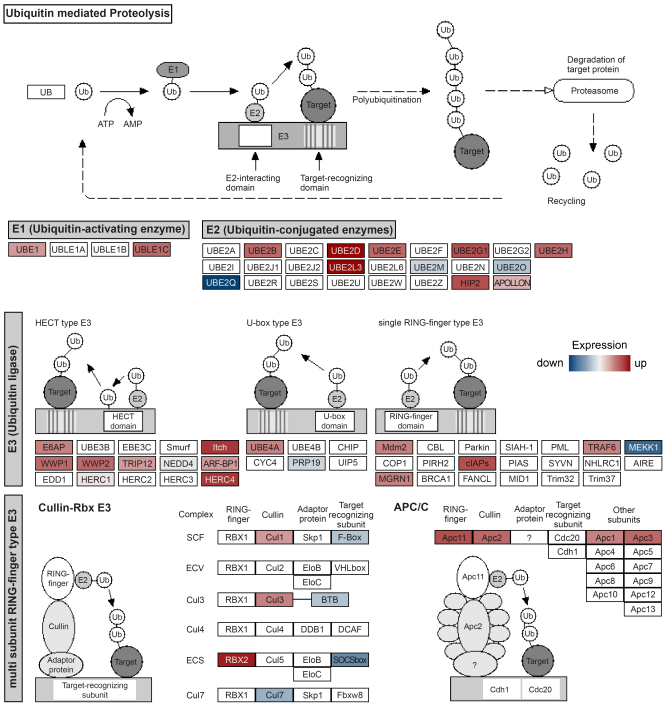

Figure 1 | **KEGG pathway for Ubiquitin mediated proteolysis (mmu04120) showing significantly DEG between 6 months old FACS-enriched *PV-Cre/tdTomato/Dnmt1* control and knockout interneurons.** Genes, which were significantly altered in expression ($P < 0.05$, Benjamini adjusted and $\log_2FC > 1$) and annotated in the KEGG pathway are labeled by a red (up-regulated in KO) to blue (down-regulated in KO) heat-map. GO analysis of all DEGs revealed a significant enrichment of this KEGG pathway ($P = 0.009996$; Benjamini adjusted, data not shown). Data obtained and analyzed as described in Pensold et al. (2020). AMP: Adenosine monophosphate; ATP: adenosine triphosphate; DEG: differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; KO: knockout; PV: parvalbumin; WT: wild-type.

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