Epigenetics

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Age-Dependent Levels of 5-Methyl-, 5-Hydroxymethyl-, and 5-Formylcytosine in Human and Mouse Brain Tissues**

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Abstract: *The absolute levels of 5-hydroxymethylcytosine (hmC) and 5-methylcytosine (mC) in human brain tissues at various ages were determined. Additionally, absolute levels of 5-formylcytosine (fC) in adult individuals and cytosine modification levels in sorted neurons were quantified. These data were compared with age-related fC, hmC, and mC levels in mouse brain samples. For hmC, an initial steady increase is observed, which levels off with age to a final steady-state value of 1.2 % in human brain tissue. This level is nearly twice as high as in mouse cerebral cortex. In contrast, fC declines rapidly with age during early developmental stages, thus suggesting that while hmC is a stable epigenetic mark, fC is more likely an intermediate of active DNA demethylation during early brain development. The trends in global cytosine modification dynamics during the lifespan of an organism are conserved between humans and mice and show similar patterns in different organs.*

In addition to the four canonical Watson and Crick bases and 5-methylcytosine (mC), it has recently been discovered that mammalian DNA also contains 5-hydroxymethylcytosine (hmC),[1] 5-formylcytosine (fC),[2] and 5-carboxycytosine[2b,3]. The biological role of these cytosine modifications is presently a focus of intense research, to which end the amount and distributions of the new bases need to be analyzed as the first step. Particularly large amounts of the base hmC were found in DNA isolated from mouse brain tissues.[1,4] In cerebral cortex, for example, the hmC level reaches up to 0.8 % with respect to all present cytosines.[4,5] Additionally, an increase in hmC content was observed during postnatal development in mice.[4,6] These findings directly lead to the question of how hmC levels in humans vary over a lifetime, since knowledge of age-dependent changes in hmC and fC levels is essential in order to gain insight into potential functions of these newly found bases.[7] So far, however, information about cytosine modification levels in human tissues is limited. Herein we report absolute cytosine modification levels in human brain tissues at fetal and different postnatal developmental stages. We compare these data with modification levels previously reported by us for adult individuals.[8] Quantification was performed by using our reported LC–MS-based isotope dilution method, in which synthetic isotopically labelled mC, hmC, and fC nucleosides are used as internal standards (Figure 1).[5c,8,9]

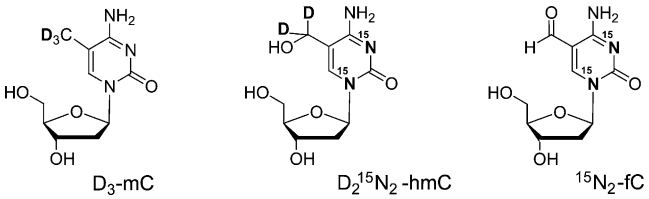

Figure 1. Depiction of the isotopically labeled mC, hmC, and fC nucleosides used as internal standards for mass spectrometry based quantification (D and ^{15}N atoms are highlighted in bold).

For analysis, brain tissues from humans of different ages were provided by the BrainBank Munich. Tissues from a 15 week old fetus (15. WOP) and from four individuals between the ages of 0.6 and 88 years were analyzed. To this end, DNA was separately isolated from grey matter (cerebral cortex) and white matter of the cerebrum. The data obtained were compiled with previously reported data from four

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individuals aged 61 (two individuals), 84, and 85.[8] In addition, from the 22 and 85 year old individuals, we were able to obtain DNA from cerebellum, again from both white and grey matter. Because prior studies with mouse tissues had revealed that cerebellum contains 40% less hmC compared to cerebrum,[4] a similar comparison in human tissues appeared highly desirable. Our quantification results regarding the absolute global levels of hmC in human brain tissues[10] are summarized in Figure 2.

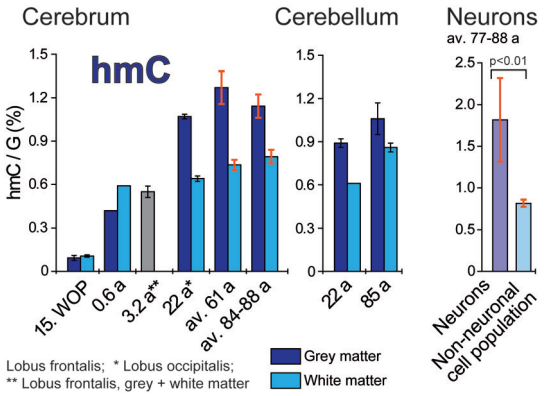

Figure 2. Age-related hmC levels in human cerebrum, cerebellum, and different brain cell populations. Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as a reference because the overall G content is equal to the sum of cytosine (C) and the cytosine derivatives mC, hmC, and fC. In cerebrum and cerebellum, the white and grey matter were analyzed separately. Data for samples 61a (two individuals), 84a, and 85a (here for cerebrum only) were taken from Kraus et al.[8] Cerebral occipital grey matter tissue of five human individuals aged 77–88 was sorted into neuronal and non-neuronal nuclei which were then analyzed separately. Black error bars represent the standard deviation of two to four technical replicates, thick red error bars represent the standard deviation of two to four biological replicates (the number of replicates was dependent on sample size). In the case of the 0.6 year old individual, owing to limited sample size, only one measurement could be performed. For statistical analysis, Student's unpaired two-tailed *t*-test was used. WOP = week of pregnancy, a = anno (year), av = average.

Our data show that in grey and white matter of the human cerebrum, the hmC level strongly increases with age. While in the cerebrum of a 15 week old fetus, only 0.1% hmC is present in both white and grey matter, the fully developed human brain shows a constant hmC level of 1.2% in the grey matter, which is rich in neuronal cell bodies.[8] This is nearly twice the level detected in mouse cerebral cortex.[4] In white matter, with myelinated axons surrounded by glial cells as the main components, hmC is less abundant. Nevertheless, the mean level of 0.75% hmC observed in adults is higher than in most mouse brain tissues.[4,8] Furthermore, in white matter the increase of hmC levels off already at the age of approximately one year. This is much earlier than in grey matter, where we observe a steady-state level only after the age of 22. Although white matter initially shows a higher hmC level compared to grey matter, this order is inverted during adolescence. Similar to cerebrum, the human cerebellum shows an age-dependent increase in hmC content, although at lower absolute levels. Our findings are in good agreement with mouse data available from others[6] and our group.[4]

We wondered whether the differences observed between the hmC levels in grey and white matter could be explained by large differences in the hmC content of neurons and glial cells. To answer this question, cerebral occipital cortex tissue samples from five individuals aged 77–88 were sorted into neuronal and non-neuronal nuclei. Cytosine modification content was then determined separately for both populations. This experiment indeed revealed a higher level of hmC in neurons compared to non-neuronal cells (1.82% vs. 0.82%; Figure 2).[10d]

In order to relate our hmC data to cytosine methylation, we next quantified the levels of mC in human cerebrum and cerebellum at early developmental stages and during adolescence,[6a, 10b–f, 11] again separately for grey and white matter. The resulting data, together with published data from four older individuals,[8] are compiled in Figure 3.

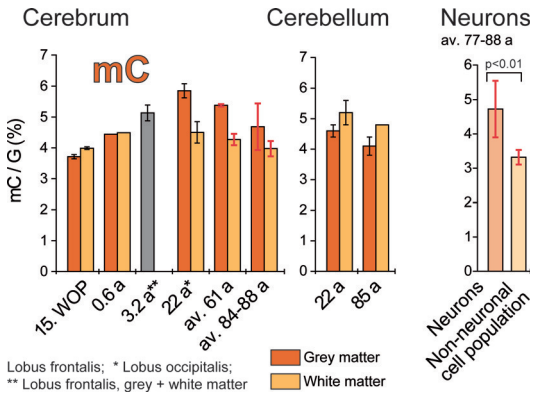

Figure 3. Age-related mC levels in human cerebrum, cerebellum, and different brain cell populations. Data for samples 61a (two individuals), 84a, and 85a (here for cerebrum only) were previously reported by us.[8] Further details as in Figure 2.

In white matter, an age-dependent change for mC is barely detectable. Here, the mC level remains roughly constant at about 4.3% during a human's lifetime. A moderate age effect is observed in the neuronal cell-body-rich grey matter, where mC values increase from 4.4% (0.6a) to a maximum of roughly 6% (22a). This finding is in full agreement with a recent study by Lister et al., who measured age-dependent de novo methylations in human and mouse lobus frontalis.[6a]

In cerebellum, no age effect is observed. In the cell-sorting experiment, an elevated mC level was observed in neurons with respect to the non-neuronal cell population (Figure 3 and the Supporting Information).[6a, 10d, 11a] In summary, our data show that global mC levels in humans change only slightly with age.

We next studied the levels of fC[2a] in human brain tissues. This base[2b] is proposed to be associated with active DNA demethylation.[1b, 2, 3, 5a, 12] Because of limited human tissue availability, in combination with the fact that more tissue is needed to detect the low levels of fC,[5c] data could only be obtained for the individuals aged 61 (sample 61*) and 85 (Figure 4).

In the cerebrum of adult human individuals, we observe fC levels of roughly $2–3 \times 10^{-4}$% fC/G, which correspond to

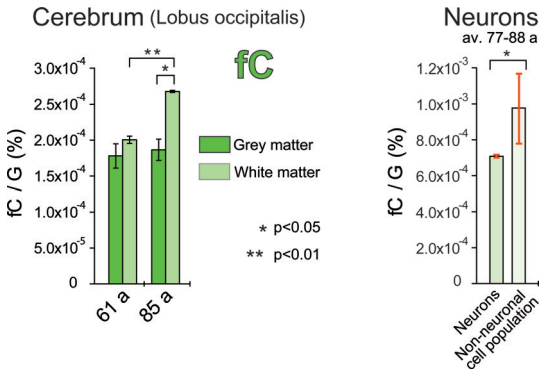

Figure 4. Global fC levels in cerebrum tissues from two human adults and in different brain cell populations. Further details as in Figure 2.

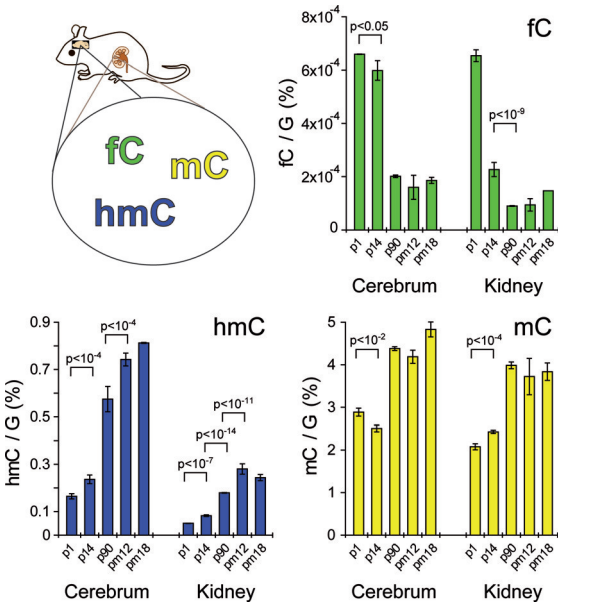

Figure 5. Global levels of fC, hmC, and mC in mouse cerebrum (cerebral cortex) and kidney at postnatal days 1 (p1) and 14 (p14) and in adult mice aged 90 days, 12 months, and 18 months. Values are given as modifications per 100 guanine bases. Guanine (G) was chosen as reference because it amounts to the sum of cytosine (C) and its derivatives mC, hmC, and fC. Samples were taken from wild-type mice (C57-BL6). Error bars represent the standard deviation of two (p1) or three (p14–pm18) biological replicates. For statistical analysis, Student's unpaired two-tailed *t*-test was used. pm = postnatal month.

$4–8\times10^{-7}$ fC modifications per nucleoside (N). Similar levels of fC were found in the grey matter of the two individuals. By contrast, the fC levels differ in white matter. Here, the 85 year old individual showed an increased fC content in white compared to grey matter, while for the individual aged 61, comparable levels of fC were detected in both tissues. Consistent with this, the cell-sorting experiment revealed that with 9.8×10^{-4}% fC/G, more fC is present in the non-neuronal cell population, while in neurons, we observed a fC level of only 7.1×10^{-4}% (see Figure 4 and the Supporting Information). It should be noted that the fC levels observed by us in human cerebrum are as high as the level of fC in adult mouse cerebrum (see also Figure 5).[5c] For a comparison of fC and hmC levels reported in this study with previously published data regarding the levels of these bases in mouse embryonic stem cells and organs at postnatal day 90, see Figures S1 and S2 in the Supporting Information.

To further study the age-dependent changes of cytosine modification levels in the mammalian brain and to overcome the limited human tissue availability, we expanded our investigations to mouse tissues. We analyzed fC, hmC,[4,6,13] and mC[6a,g,11c] levels in cerebrum tissue (cerebral cortex) sampled at postnatal day 1 (p1, newborn) and day 14 (p14), and from adult mice at postnatal day 90 (p90), postnatal month 12 (pm12), and postnatal month 18 (pm18; Figure 5).

Besides the expected increase in hmC content with age, we surprisingly observe a strong decrease in the fC modification level at early developmental stages.[9b] Because fC is proposed to be generated from hmC by Tet-induced oxidation,[2b] the decreasing fC level does not fit with the increasing amounts of hmC during brain development. One possible explanation for this counterintuitive observation is that fC is indeed an intermediate of an active DNA demethylation process[1b,2,3,5a,12] and that active demethylation events in the brain cease with age. The higher fC levels in early lifetime suggest a faster hmC turnover via oxidation to fC,[2b] followed by active demethylation.[2,3,5a,12d,e] With increasing age, these as yet ill-defined processes may slow down, which would lead to the observed accumulation of hmC.

We observe similar trends in mouse kidney tissue, which was previously found to contain intermediate levels of hmC.[5a] Here too, a low hmC level in young animals corresponds to a high fC level, while higher hmC levels during later developmental stages go hand in hand with low fC values. This observation is in good agreement with the idea that reduced active demethylation is a reason for hmC accumulation in tissues during lifetime.

Finally, the mC level observed in mouse cerebrum (cerebral cortex) remains roughly constant until p14. It then shows a much stronger increase with age compared to human cerebrum.

In summary, we provide the first data on age-dependent variations in global hmC levels in humans over the entire lifespan. After a strong increase during early postnatal stages and adolescence, hmC reaches a steady-state level of 1.2% in the fully developed brain.[8] This is nearly twice as high as in mouse cerebral cortex.[4] Furthermore, global levels of fC in brain tissues of human adults, and cytosine modification levels in human cerebral occipital cortex neurons were quantified. We also report age-dependent variations in fC, hmC, and mC levels in mouse tissues between postnatal day 1 and postnatal month 18. Our measurements revealed that the level of fC decreases strongly at early developmental stages, showing an inverted age-dependent trend compared to hmC. Our data support the idea that fC in the developing brain is mainly linked to active DNA demethylation,[2,3,5a,12d,e] while hmC in this context is rather a stable epigenetic mark.[14] The observed trends in global cytosine-modification dynamics during the lifespan of an organism are conserved[15] between mammalian species[16] and are similar in appearance in different organs.[4,6,9b,10a,b,e,11,16,17]

Keywords: 5-formylcytosine · 5-hydroxymethylcytosine · aging · epigenetics · cerebral cortex

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