Aneuploidy in the human brain and cancer
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Chapter 2

Does Aneuploidy in the Brain Play a Role in Neurodegenerative Disease?

Hilda van den Bos, Diana C.J. Spierings, Floris Foijer, and Peter M. Lansdorp

Abstract
Aneuploidy, a state in which cells exhibit copy number changes of (parts of) chromosomes is a hallmark of cancer cells and, when present in all cells, leads to miscarriages and congenital disorders, such as Down syndrome. In addition to these well-known roles of aneuploidy, chromosome copy number changes have also been reported in some studies to occur in neurons in healthy human brain and possibly even more in Alzheimer’s disease (AD). However, the studies of aneuploidy in the human brain are currently under debate as earlier findings, mostly based on in situ hybridization approaches, could not be reproduced by more recent single cell sequencing studies with a much higher resolution. Here we review the various studies on the occurrence of aneuploidy in brain cells from normal individuals and Alzheimer’s patients. We discuss possible mechanisms for the origin of aneuploidy, the pros and cons of different techniques used to study aneuploidy in the brain and we provide a future perspective.
Introduction

Aneuploidy is a state in which cells have an abnormal and unbalanced number of chromosomes. An aneuploid cell can have one or more extra chromosomes, called hyperploid, or it can have lost one or more chromosomes, which is called hypoploid. Following this definition of aneuploidy, a cell that has doubled its complete genome without dividing is called tetraploid and not aneuploid, because a balanced genome is still present.

Aneuploidy is well known from cancer and systemic trisomies such as Down syndrome. Indeed, at least 2 out of 3 cancers exhibit aneuploidy \(^1\)–\(^3\). Although it has been shown that aneuploidy causes stress and reduces cellular fitness \(^4\)–\(^7\), cancer cells have somehow found a way to cope with aneuploidy and manage to proliferate despite the detrimental consequences of aneuploidy. This is known as the aneuploidy paradox \(^6\). Perhaps by selecting numerical chromosomal abnormalities that promote tumor progression in addition to other structural genomic rearrangements, cancer cells can survive and keep growing \(^8\),\(^9\). The profound effect that aneuploidy has on healthy cells is emphasized by the fact that, besides sex-chromosome abnormalities, in humans only three systemic autosomal trisomies are compatible with life: trisomy 21 causing Down syndrome, trisomy 13 causing Patau’s syndrome and trisomy 18 causing Edward’s syndrome \(^10\)–\(^12\). The viability of these systemic aneuploidies can probably be explained by the fact that these three chromosomes contain the lowest number of genes of all human autosomes. Even though these trisomies can be compatible with life, the majority of such trisomic pregnancies end with a miscarriage, and the children that do survive until birth suffer from severe cognitive and developmental defects \(^13\).

But what is the origin of aneuploid cells? Aneuploidy is the result of chromosomal instability (CIN) and can arise when errors occur during DNA replication or mitosis. To prevent such errors, cells have evolved many checkpoints and mechanisms that ensure faithful replication of DNA and proper chromosome segregation. One of these checkpoints, the spindle assembly checkpoint (SAC), ensures that chromosome segregation is prevented until all chromosomes are properly attached to the mitotic spindle. Therefore, when the SAC fails, daughter cells can end up with gained or lost chromosomes. Furthermore, merotelic attachments – chromosome attachments where one of the sister chromatids is attached to both spindle poles – can result in aneuploidy even with a functional SAC. Finally several other mechanisms, such as cohesion defects, multipolar spindles and lagging chromosomes, can all lead to incorrect chromosome segregation and thus aneuploidy \(^14\).

Many tumor cells have inactivated the tumor suppressor p53, a key transcription factor in the DNA damage response and other cell cycle checkpoints. When functional, stresses such as DNA damage lead to activation of p53. P53 then induces a cell cycle arrest and activates DNA repair or induces apoptosis when the damage cannot be repaired. Loss of p53 makes cells more tolerant of aneuploidy \(^15\) and allows them to propagate despite DNA damage or short telomeres \(^16\).

When telomeres become too short following proliferation or due to defects in telomere function cells exit the cell cycle \(^17\). Loss of p53 overcomes this tumor suppression mechanism and allows cells to proliferate with critically short telomeres. This results in end-to-end fusion of sister chromatid telomeres, resulting in dicentric chromosomes. Dicentric chromosomes are likely to missegregate during mitosis thus resulting in aneuploidy and DNA breaks. Such broken chromosomes can trigger a so-called breakage-fusion-bridge (BFB) cycle, which can
Chapter 2

The role of aneuploidy in neurodegenerative disease

Aneuploidy during development and aging

Studying aneuploidy in the brain is complicated by the largely post-mitotic state of adult neurons, limiting the methods that can be used. Therefore, many studies have used methods like interphase FISH, or DNA dyes such as DAPI or PI in combination with for example flow cytometry to determine the DNA/genome content of individual cells. Given the detrimental effect that aneuploidy has on cells, one would expect somatic cells of the brain to be perfectly euploid. A publication by Rehen et al. in 2001 challenged this view. In this study, the authors quantified aneuploidy in embryonic mouse neuroblasts, adult cortex and lymphocytes using spectral karyotyping (SKY) and fluorescence in situ hybridization (FISH). They found ~33% of the 220 mouse neuroblast metaphase cells studied to be aneuploid as assessed by SKY, the great majority of which was hypoploid (98%). In contrast, of the adult mouse lymphocytes only 3% of the metaphase cells were identified as being aneuploid. In the same study, X and Y chromosome aneuploidy was assessed using FISH in adult mouse brain. They found X or Y chromosome aneuploidy occurring in 1.2% of the brain cells examined. The same rate of aneuploidy was found when comparing total adult nuclei with nuclei ≥ 10µm, which are likely to be neurons. In comparison, the rate of X and Y chromosome aneuploidy in the mouse neuroblasts was ~10% (of which ~8% loss and 2% gain). In summary, these results suggest a high rate of aneuploidy in the developing mouse brain, and a much lower but still significant number of aneuploid cells in the adult mouse brain. A number of other studies reported similar aneuploidy rates in the developing human brain using interphase FISH. Aneuploidy rates up to 30-35% in the (developing) human brain were found, some studies reporting mainly chromosome losses, another mainly chromosome gains. The cause of aneuploidy in the developing brain was speculated to be mitotic segregation defects, since in dividing mouse neuronal progenitor cells lagging chromosomes and multipolar spindles have been found. In contrast, there is little consistency in the aneuploidy rates reported in adult human brain. For example, the percentages of aneuploidy range from 0 up to 40: no aneuploidy was found in 2 normal brains (n = 200/chromosome/sample), ~4% aneuploidy of chromosome 21 (n = 500-1000 per sample), 1.3-7.0% aneuploidy per chromosome (n ≥ 500 for adult and ≥ 1000 for embryonic samples for each chromosome) and 40% aneuploidy in the normal human brain (n = NA). All of these studies used FISH to count the chromosomes. A study performed by the group of Rehen, which combined several techniques, reported that aneuploid neurons seem to be integrated into the brain circuitry like euploid cells and that aneuploid neurons can be activated and seem to be functional. Taken together, although the rate of aneuploidy reported varies widely, most reports state that, especially in the developing brain, aneuploid cells are present at detectable frequencies in the normal brain.

But if aneuploid cells are present in large numbers in the developing brain, and in lower quantities in the adult human brain, what happens during aging? An increase in aneuploidy for chromosome 17 and 21 was found in the hippocampus of aged individuals compared to young controls. In sharp contrast, another study determined the number of cells with a DNA
content above the diploid level in brain samples ranging from 30 - 90 years of age. They found a decrease in the number of cells exceeding the diploid level with age 30, but suggested that this might be due to a biased selection of ‘healthy aging’ brains. Taken together, there appears to be little consensus on whether aneuploid cells are present in adult brains, their frequency, and changes during aging. An overview of previous studies on aneuploidy in the brain is shown in Table 1. To explain the high rates of aneuploidy in the brain, several of the above-discussed studies hypothesized that aneuploidy in fact might contribute to neuronal diversity. The human brain consist of approximately 100 billion neurons forming an estimated 0.15 quadrillion ($10^{15}$) synapses and there is a very high diversity of neurons 31. Human brains have a high level of cellular heterogeneity and it has been estimated that our brains might have as many as 10.000 different types of neurons 32. All these different neurons work together to allow us to perform complex tasks. It is suggested that the presence of aneuploid neurons could be one of the mechanisms providing more variability and complexity to the human brain 14,32–34.

**Origin of aneuploid cells in the brain**

If our brain indeed contains aneuploid cells, where do they originate? As discussed above, aneuploid cells are usually formed when something goes wrong with DNA replication or in mitosis. Aneuploid cells could therefore be generated during early development when there is a high rate of cell division, or later in life during normal or abnormal cell division. We can think of a number of explanations. First, since especially in the developing brain high rates of aneuploid cells have been found, defective clearance of these cells could explain their presence in the adult brain 47. During brain development many more cells are formed than end up in the adult brain suggesting the existence of strong selection for certain cell types 48. This process possibly includes negative selection for aneuploid cells, which could explain the much lower rate of aneuploidy reported in the adult brain than in the developing brain. Failure to select for diploid cells during this selection could result in aneuploid cells being present in the adult brain 36,49. Indeed, *in vitro* experiments have shown that the differentiation of pluripotent stem cells into neural progenitor cells by retinoic acid (RA) is accompanied by increased levels of aneuploidy and micronuclei 50. Second, it has been hypothesized that cell cycle re-entry and failure to complete the cell cycle of neurons might be involved in neurodegeneration 37,51–53. Neurons might attempt to re-enter the cell cycle, replicate their DNA but fail to complete cell division. The main evidence for this hypothesis is the observation that post-mitotic neurons in AD brains sometimes stain positive for cell cycle markers such as PCNA, cyclins and cyclin depended kinases (CDKs) 54–60. As a consequence of re-entering the cell cycle, the presence of tetraploid cells in the brain is expected. These cells have completed DNA replication but are unable to complete mitosis. But whether tetraploid cells are indeed present in the brain is still under debate 35,40. By counting fluorescent signals from probes directed at either chromosome 11, 18 or 21, Yang et al., found that 3.7% of the hippocampal cells in 6 AD brains have displayed 3 or 4 fluorescent signals. Although the fluorescent probes were not combined on individual cells, no distinction was made between 3 and 4 fluorescent signals and no neuronal marker or DNA counter stain was used, the researchers conclude from these results that 3.7% of the hippocampal cells in these AD brains has a fully or partially replicated genome. But these results can also reflect single chromosome aneuploidies 35.
Table 1. Overview of studies on aneuploidy in the brain

<table>
<thead>
<tr>
<th>Species/Cell type</th>
<th>Technique(s) used</th>
<th>Chromosomes studied</th>
<th>Main conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse neuroblasts and adult cortical cells</td>
<td>SKY, FISH, FACS</td>
<td>All chromosomes</td>
<td>~33% aneuploidy in neuroblasts, of which 98% hypoploidy, 1.2% X/Y aneuploidy in adult cortical cells</td>
<td>Rehen et al., 2001</td>
</tr>
<tr>
<td>Undiseased human prefrontal cortical (area 10) neurons</td>
<td>FISH</td>
<td>1, 7, 8, 13, 16, 18, 21, 22, X and Y</td>
<td>No aneuploidy found</td>
<td>Yurov et al., 2001</td>
</tr>
<tr>
<td>Human hippocampal pyramidal cells of Alzheimer's disease patients and age matched controls</td>
<td>FISH</td>
<td>11, 18 and 21</td>
<td>3 or 4 hybridization spots in 3.7% of cells in AD, no cells with more than 2 hybridization spots in controls</td>
<td>Yang et al., 2001</td>
</tr>
<tr>
<td>Mouse neuronal progenitor cells</td>
<td>SKY</td>
<td>All chromosomes</td>
<td>33.2% aneuploidy</td>
<td>Yang et al., 2003</td>
</tr>
<tr>
<td>Mouse subventricular zone (SVZ) cells</td>
<td>DAPI staining, SKY</td>
<td>All chromosomes</td>
<td>33% aneuploidy in SVZ cells, of which ~76% hypoploidy with the majority having lost multiple chromosomes</td>
<td>Kausal et al., 2003</td>
</tr>
<tr>
<td>Human neurons and non-neuronal brain cells</td>
<td>FISH</td>
<td>21</td>
<td>~4% aneuploidy of chromosome 21, mean chromosome number of 2.05, no difference between neurons and non-neuronal cells</td>
<td>Rehen et al., 2005</td>
</tr>
<tr>
<td>Mouse cortical neurons</td>
<td>FISH</td>
<td>X and Y</td>
<td>~0.2% combined hyperploidy</td>
<td>Kingsbury et al., 2005</td>
</tr>
<tr>
<td>Human (undiseased and AD) and mouse neurons</td>
<td>FISH</td>
<td>Not stated</td>
<td>43% (32-53%) aneuploidy in AD neurons, 40% (38-47%) in undiseased neurons, similar degree in murine neurons (data not shown)</td>
<td>Pack et al., 2005</td>
</tr>
<tr>
<td>Human brain cells from fetal tissue (medulla oblongata) and adult cortex (area 10)</td>
<td>FISH</td>
<td>1, 13/21, 18, X and Y</td>
<td>0.6-3.0% aneuploidy per chromosome in fetal brain cells, 0.1-0.8% aneuploidy per chromosome in adult brain cells</td>
<td>Yurov et al., 2005</td>
</tr>
<tr>
<td>Human entorhinal cortical neurons from patients with AD and controls</td>
<td>SBC, CISH</td>
<td>Overall DNA content and 17</td>
<td>Increased hyperploidy in AD, increased hybridization spots for chromosome 17 in AD</td>
<td>Mosch et al., 2007</td>
</tr>
<tr>
<td>Human fetal brain</td>
<td>FISH</td>
<td>1, 9, 15, 16, 17, 18, X and Y</td>
<td>1.25-1.45% aneuploidy per chromosome</td>
<td>Yurov et al., 2007</td>
</tr>
<tr>
<td>Human buccal and hippocampal cells from AD patients and controls</td>
<td>FISH</td>
<td>17 and 21</td>
<td>Increased aneuploidy in buccal cells of AD patients but not in hippocampus</td>
<td>Thomas et al., 2008</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Species/Cell type</th>
<th>Technique(s) used</th>
<th>Chromosomes studied</th>
<th>Main conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse NPCs and human and mouse cerebellum</td>
<td>DAPI staining, FISH</td>
<td>Mouse: 16 and X, Human: 6 and 21</td>
<td>15.3% aneuploidy in mouse NPCs at P0, 20.8% at P7, 0.5-1.0% aneuploidy per chromosome in adult mouse and human NeuN+ and NeuN-cerebellar nuclei</td>
<td>Westra et al., 2008</td>
</tr>
<tr>
<td>Cerebral cortex of normal human brain and AD patients</td>
<td>FISH</td>
<td>1, 7, 11, 13, 14, 17, 18, 21, X and Y</td>
<td>0.5% aneuploidy per chromosome in normal and AD brain, except increased chromosome 21 aneuploidy in AD: 6-15%</td>
<td>Yurov et al., 2009</td>
</tr>
<tr>
<td>Cortical and hippocampal nuclei of normal human brain and AD patients</td>
<td>FISH</td>
<td>4, 6 and 21</td>
<td>0.4-3.5% tetrasy in non-neuronal cells No difference in normal and AD brain in non-neuronal cells, no tetrasy in neurons</td>
<td>Westra et al., 2009</td>
</tr>
<tr>
<td>Entorhinal cortex of normal, preclinical AD, mild AD and severe AD patients</td>
<td>SBC, FISH, CISH</td>
<td>Overall DNA content and 17</td>
<td>10% hyperploidy in normal brain, ~27% in preclinical AD, ~35% in mild AD and ~23% in severe AD</td>
<td>Arendt et al., 2010</td>
</tr>
<tr>
<td>Cerebral and cerebellar cortex of young and old mice</td>
<td>FISH</td>
<td>1, 7, 14, 15, 16, 18, 19 and Y</td>
<td>1% aneuploidy per chromosome in cerebral cortex of young mice, 2.3% in old mice, no increase in aneuploidy with age in cerebella</td>
<td>Faggioli et al., 2012</td>
</tr>
<tr>
<td>Neurons and NPCs derived from human induced pluripotent stem cells and normal human frontal cortex</td>
<td>Single cell sequencing, FISH</td>
<td>All chromosomes 20 and X with FISH</td>
<td>27.5% aneuploidy in hiPSC-derived neurons, 5% in hiPSC-derived NPCs, 2.7% aneuploidy in normal frontal cortex</td>
<td>McConnell et al., 2013</td>
</tr>
<tr>
<td>Prefrontal cortex of normal brain and AD patients</td>
<td>FISH, FISH</td>
<td>1, 7, 11, 16, 17, 18 and X</td>
<td>Increased X chromosome aneuploidy in AD (1.16-1.74% in controls, 2.78-4.92% in AD)</td>
<td>Yurov et al., 2014</td>
</tr>
<tr>
<td>Human cortical neurons</td>
<td>Single cell sequencing</td>
<td>All chromosomes</td>
<td>5% aneuploidy in normal human cortical neurons</td>
<td>Cai et al., 2014</td>
</tr>
<tr>
<td>Mouse embryonic NPCs and adult brain, human frontal cortex</td>
<td>Single cell sequencing</td>
<td>All chromosomes</td>
<td>No aneuploidy in mouse NPCs and neurons, 2.3% aneuploidy in adult mouse brain, 2.2% aneuploidy in human brain</td>
<td>Knouse et al., 2014</td>
</tr>
<tr>
<td>Mouse embryonic and adult cerebral and cerebellar cortex</td>
<td>FISH</td>
<td>1, 7, 18</td>
<td>~1% (cerebral) and 0.1% (cerebellar) aneuploidy per chromosome in 14 weeks and 6 month old mice, ~30% aneuploidy per chromosome (chr. 1 and 18) in embryonic mouse brain</td>
<td>Andriani et al., 2016</td>
</tr>
<tr>
<td>Prefrontal cortical neurons of normal brain and AD patients</td>
<td>Single cell sequencing</td>
<td>All chromosomes</td>
<td>No increased aneuploidy in AD: 0.7% aneuploidy in controls, 0.6% in AD</td>
<td>van den Bos et al., 2016</td>
</tr>
</tbody>
</table>
In contrast, a study performed by Westra et al. failed to find any tetraploid neurons in the cells studied \(^{40}\), the only cells with 4 fluorescent signals were non-neuronal and no difference was found between AD and control samples. Also, this hypothesis of aberrant cell cycle re-entry is not supported by the single chromosome aneuploidies found of which, in most cases only one copy of one chromosome is lost or gained in a cell. Third, the limited amount of neurogenesis taking place in the adult brain could potentially be a source of aneuploid neurons \(^{50,61}\). In summary, aneuploid neurons in the adult brain can have originated in the developing brain and escaped clearing mechanisms, or formed due to cell cycle re-entry and failed mitosis of adult neurons although the evidence for this hypothesis is contrasting.

**Aneuploidy in neurodegeneration**

Because human brain tissue is inaccessible in vivo, many researchers used peripheral cells, such as lymphocytes and fibroblasts, to study the correlation between genomic damage and neurodegenerative diseases such as AD. Several studies with conflicting results have been published: some show a correlation between AD and increased peripheral aneuploidy \(^{62-66}\), while others report no difference \(^{67,68}\). Counting the presence of micronuclei is a way to assess genome stability. Micronuclei are formed when chromosome segregation is flawed, causing a part of or a whole chromosome to end up outside of the nucleus in a so-called micronucleus. Therefore, the number of micronuclei present is a marker for chromosome missegregation. Interestingly, AD patients were found to have increased numbers of micronuclei in their lymphocytes, mostly containing whole chromosomes \(^{69}\). More specifically, AD patients were reported to have increased rates of trisomy 21 in lymphocytes, while missegregation rates for chromosome 13 were unaltered, when compared to healthy controls. \(^{70}\) Similarly, patients suffering from (AD) were found to exhibit frequent copy number changes for chromosomes 17 and 21 in buccal cells. \(^{29}\)

Since neurons are post-mitotic, methods requiring dividing cells to determine chromosome copy numbers cannot be used when studying aneuploidy in neurons. Most studies therefore make use of fluorescence in situ hybridization (FISH) based methods to count chromosomes in brain cells. When comparing control brain with early and late AD samples using slide-based cytometry (SBC), PCR amplification of alu repeats, and chromogenic in situ hybridization (CISH), a 2 fold increase in neurons with a DNA content between 2n and 4n was found \(^{37}\). Also in preclinical stages of AD an increased number of neurons with a more than diploid DNA content has been reported \(^{41}\). Iourov et al, found no overall significant difference in aneuploidy rates when looking at copy number changes of 7 autosomes (chromosomes 1, 7, 11, 13, 14, 17 and 18) and the X and Y chromosome. But a specific increase in chromosome 21 aneuploidy in neurons of AD brain samples was identified, of which 60% where gains and 40% loss of chromosome 21 \(^{39}\). On the other hand, in a recent study a 2 fold increase in X chromosome aneuploidy was found in AD neurons when compared to age matched controls \(^{43}\). To summarize, although again the rates of aneuploidy and which chromosomes are affected differ between studies, the overall trends suggests aneuploidy might be increased in AD \(^{46}\).

**The possible link between Down syndrome and Alzheimer’s disease**

Down syndrome is the most common autosomal systemic aneuploidy. Besides the observation of increased levels of trisomy 21 in the brains of AD patients, Down syndrome and
AD have more in common. First, Down syndrome patients are much more likely to develop AD and at an earlier age than genetic euploid individuals. This could be related to the fact that the amyloid precursor protein (APP) gene, mutations in which are known to cause early onset AD, is located on chromosome 21. Also, in the brains of individuals with Down syndrome over 40 years of age protein aggregates, plaques and tangles, are present in amounts that are also observed in AD patients brains. On the other hand, not all patients with trisomy 21 over 40 develop AD, although all of them develop plaques and tangles. Second, it has been found that young mothers (<35 years) of a child with Down syndrome have increased chromosomal instability, as shown by having more micronuclei, and more chromosomal missegregation events in their lymphocytes. In the great majority of cases (95%) the extra chromosome 21 originates from a maternal nondisjunction event. Moreover, Schupf et al., found that young mothers of a child with Down syndrome have a 5 fold increased risk to develop AD, while the risk was not increased in mothers who had a child with Down syndrome at a later age (>35 years). It is therefore hypothesized that some women might have a genetic susceptibility to chromosome nondisjunction, increasing the risk of both getting a child with Down syndrome as well as developing AD. Lastly, also mouse models for Down syndrome display characteristics of AD. For example, the widely used mouse model Ts65Dn, which has an extra copy of a large part of Mmu16, the mouse homolog of a large part human chromosome 21 including APP, displays increased levels of APP and Aβ, as well as progressive memory decline and neurodegeneration in adult mice.

How can aneuploid cells play a role in neurodegeneration?
Aneuploidy was shown to reduce cellular fitness. It was therefore suggested that aneuploid cells might be selectively affected by cell death in the brains of AD patients. According to this hypothesis, a decrease in aneuploidy rates might be expected as the disease progresses. This is in line with the observation by Arendt et al. of decreased hyperploidy in severe AD compared to mild AD. It must be noted that in this study the total amount of DNA was studied with a DNA dye, rather than the rate of aneuploidy. On the other hand, if aneuploid cells remain present in the aging brain, aneuploidy could contribute to neurodegenerative diseases through proteotoxic stress. Misfolding of proteins leads to proteotoxic stress, the formation of protein aggregates and possibly neurodegeneration. Being aneuploid is a heavy burden for a cell. Having an extra copy of a chromosome generally means that the genes on this chromosome are transcribed and translated at the same rate compared to the two ‘normal’ copies. Therefore the cell has to deal with this 50% extra mRNA and protein. All these extra proteins have to be folded into the right conformation, or processed by the protein degradation machinery. This leads to increased pressure on chaperones and the protein degradation machinery. Since protein aggregates are thought to play an important role in the development and progression of many neurodegenerative diseases, their formation might be stimulated by excess proteins that overload the protein folding and degradation machinery. Trisomy 21 has been reported to be more prevalent in the brains of AD patients. The extra copy of the APP gene on chromosome 21, which encodes the β-amyloid protein, could trigger the formation of amyloid plaques resulting in proteotoxic stress and ultimately cell death.
Chapter 2

Low levels of aneuploidy found in the brain using single cell sequencing.

Recently, it became possible to use single cell next generation sequencing (NGS) to look at aneuploidy in individual cells (Figure 1)\textsuperscript{42,44}. Compared to the classic method for measuring aneuploidy using FISH, single cell sequencing has some important advantages\textsuperscript{85}. First, FISH studies are in most cases limited to examining only a few chromosomes per cell. Therefore the total rate of aneuploidy is usually determined by extrapolating the aneuploidy rates of the few chromosomes that are studied, possibly resulting in an over- or underestimation of the frequency of aneuploidy. With single cell sequencing the copy number of all chromosomes in each single cell can be determined more accurately. Each chromosome is sampled thousands of times, whereas with FISH the chromosomes are usually measured only once or twice. Although spectral karyotyping (SKY) can also be used to count all chromosomes within a cell, this method requires metaphase chromosomes and thus dividing cells, while single cell sequencing can be performed on non-dividing cells.\textsuperscript{86} Moreover, SKY is more likely to overestimate chromosome loss, due to chromosomes being washed away from the slide onto which they were dropped. This could explain the high rates of hypoploid cells found using SKY\textsuperscript{87}. Second, since with FISH the karyotype is determined by simply counting the number of fluorescent spots, in several ways this can lead to errors in chromosome counts. Failure of the probe to hybridize and non-specific binding results in overestimation of aneuploidy rates.

Fortunately, the development of single cell sequencing protocols has allowed studies of all chromosomes in single, non-dividing cells. For this approach, libraries are made of individual cells or nuclei. In most cases, library preparation starts with a whole genome amplification step. This can be problematic because uneven amplification of genomic DNA may result in a sequencing bias. Next, the DNA is fragmented either mechanically, such as by sonication, or enzymatically, for example with restriction enzymes. To enable binding of the fragments to the sequencing flow cell, adapters are ligated to either end of the fragmented DNA. Also, individual barcodes can be introduced to allow pooling (multiplexing) of more than one library on a flow cell, thus significantly reducing sequencing costs. After sequencing, the individual reads are split into libraries for each individual cell based on the cellular barcode (demultiplexing), and the copy numbers of individual chromosomes can be determined by comparing the read density on each chromosome. An extra copy of a chromosome is expected to result in 50% increase in read density, while loss of a chromosome leads to a 50% reduction of the read density on that chromosome\textsuperscript{46,88,89}. Depending on the sequencing depth, single cell sequencing can, in addition to whole chromosome aneuploidies also reveal smaller copy number changes. Since single cell sequencing is often combined with FACS sorting of single nuclei, micronuclei will be lost when sorting nuclei. Also, this method is relatively expensive and thereby limits large scale sequencing projects. Even though only few studies so far used next generation sequencing-based to karyotype cells, the results are contrasting some of the earlier FISH-based findings in that the rate of aneuploidy found was in general much lower than was reported previously. For instance, Knouse et al., identified one aneuploid brain cell of the 43 sequenced cells and all of the 9 neurons sequenced were euploid\textsuperscript{45}. Another study found 5 neurons to be aneuploid out of the 100 neurons that passed the quality criteria\textsuperscript{44}. Also, only one chromosomal gain and 2 losses were identified in 110 sequenced frontal cortex neurons of 3 individuals\textsuperscript{42}. Finally, the largest study determined aneuploidy rates in post-mortem frontal cortex neurons of normal human brain and samples from patients affected
with AD. Interestingly, a very limited number of aneuploid neurons was found; less than 1% aneuploidy both in controls and AD. All of these single cell sequencing studies use cells of which the chromosome copy numbers are known as validation of the method; human male trisomy 21 fibroblasts, human male trisomy 18 neurons, mouse trisomy 16 brain cells, and human female trisomy 21 neurons. In each case, the known aneuploidy as well as the correct number of X chromosomes, male or female, was detected with 100% accuracy, confirming the sensitivity of single cell sequencing. Studying aneuploidy in the developing human brain with single cell sequencing remains to be done. But also here, the lack of aneuploidy reported in the 36 mouse neuronal progenitor cells sequenced might be an indication that also the embryonic aneuploidy levels have been overestimated. Taken together, the results of single cell sequencing studies are in sharp contrast to the previously reported aneuploidy rates. How can these conflicting results obtained with different techniques be explained? As mentioned before, studies of aneuploidy in the human brain are complicated. Selecting a tissue or cell type as valid control is difficult, as no tissue is similar to brain tissue. Usually, lymphocytes are used as control. This potentially introduces problems, as the isolation of cells or nuclei from such very different sources requires very different experimental approaches: lymphocytes are isolated as single, unattached cells, while brain tissue needs some sort of mechanical or enzymatic dissociation to obtain individual cells or nuclei. On the other hand, brain tissue sections can also be used, but in this case the inevitable cuts through nuclei can give rise to incorrect chromosome counts. While differences in handling of the tissue or cells may explain some of the reported differences, this explanation does not apply when comparing aneuploidy in normal and diseased brain samples.

**Figure 1.** Single cell sequencing of a female cell with trisomy of chromosome 21 (A), and a male diploid cell (B). Plots are made using Aneufinder.
Chapter 2

Conclusion
The frequency of neuronal aneuploidy in the normal healthy brain remains a matter of debate. Although many studies report a certain level of aneuploidy, this is not confirmed by more recent reports using single cell sequencing. Whether the number of aneuploid cells is increased or decreased with aging and in neurodegenerative diseases remains to be conclusively shown. Aneuploid neurons could be involved in neurodegeneration because an incorrect karyotype could cause proteotoxicity via protein misfolding and aggregation. Single cell sequencing is a promising tool to address questions about aneuploidy in the brain and should provide more definite answers in the years to come.

References
The role of aneuploidy in neurodegenerative disease


Chapter 2 The role of aneuploidy in neurodegenerative disease

The role of aneuploidy in neurodegenerative disease

Chapter 3

Single-cell whole genome sequencing reveals no evidence for common aneuploidy in normal and Alzheimer's disease neurons

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