Loss of deep cerebellar nuclei neurons in the 3xTg-AD mice and protection by an anti-amyloid β antibody fragment

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Abbreviations: AD, Alzheimer disease; DCN, deep cerebellar nuclei; GCs, granule cells; PCs, Purkinje cells; scFv, single chain variable fragment

Introduction

The World Health Organization and Alzheimer Disease International have estimated that 36 million people were living with dementia in 2011, with Alzheimer disease (AD) being the most common form of dementia.1 They estimated that there are 7.7 million new cases of dementia each year and that 115 million people may be living with dementia by 2050. As a consequence, AD is considered to be a first century pandemic.

Neuritic plaques composed of amyloid β (Aβ) peptides, as well as neurofibrillary tangles of hyperphosphorylated tau protein, are the histopathological hallmarks of AD.2 Although brain regions are the structures primarily affected in AD, the occurrence of diffuse Aβ deposits has also been reported in the molecular layer of the cerebellar cortex and, to a lower extent, in the granule cell layer.3-5 Additionally, Purkinje cell (PC) loss and synaptic dysfunction are heightened as the disease progresses.5-9

The triple-transgenic mouse model of AD (3xTg-AD), harboring two human transgenes causing early onset AD (PS1M146V and APPswe) and a mutant form of tau (tauP301L), mimics both Aβ plaques and tau neurofibrillary tangles following a regional and temporal involvement homologous to humans.10 At four months of age, Aβ accumulates in cortical regions and initiates progression to the hippocampus, whereas tau hyperphosphorylation develops after Aβ accumulation (at ~12–15 mo), beginning in limbic structures and extending later through cortical regions.10-12 Because of the recognized need for early intervention in AD,13 our studies were performed with 5 mo old animals. Females were selected because they exhibit significantly greater Aβ burden and larger behavioral deficits than age-matched males.14

We recently used this model to show that immunotherapy with an anti-Aβ antibody fragment is a potential tool for the treatment of AD.15 Specifically, we constructed a single-chain variable fragment (scFv), scFv-h3D6,16 from the humanized monoclonal antibody AAB-001 (mAb-h3D6, bapineuzumab).17 An advantage of the use of such fragments compared with the use of complete antibodies is that Fc-mediated activation of microglia cannot occur. Therefore, use of scFv-h3D6 could avoid some of the undesirable effects reported in Phase 3 clinical trials for bapineuzumab.18 In fact, a single intraperitoneal dose of scFv-h3D6 reversed BPSD-like behaviors, improved long- and short-term learning and memory deficits, globally decreased Aβ-oligomers in the cortex and olfactory bulb and recovered the non-pathological levels of some apolipoproteins in the cortex (apoE and apoJ);15 however, no differences in the concentration of these molecules were found in the cerebellum.

Since involvement of human cerebellum has been described in late phases of the disease, we wondered when neuronal vulnerability
in the 3xTg-AD cerebellum, either in the cortex or the deep nuclei, could appear. A dramatic death in young 3xTg-AD deep cerebellar nuclei neurons was found and, more exciting, we successfully tested the capability of scFv-h3D6 for preventing it.

Results

The numbers of granule cells (GCs) and Purkinje cells (PCs), as well as cellular density, in each cerebellar cortex region analyzed were similar between 3xTg-AD and NTg mice (Table 1). However, differences were found between the area of GCs’ layer at the vermis of the untreated (or treated) NTg and that of the untreated 3xTg-AD animals (Table 1), which showed a mild, although significant, decrease. It is noteworthy that scFv-h3D6 treatment exerted a protective effect on the size of GCs’ layer because no differences were found between treated 3xTg-AD group and NTg groups.

Similarly, the areas of the fastigial and interpositus nucleus were smaller in the 3xTg-AD mice than in the NTg one, but no differences were found in the dentate nuclei (Table 1). These differences also occurred, and in the same direction, when both cell number and cell density were compared (Table 1). Because changes are bigger in the cell number than in the areas, we refer herein to the total number of cells rather than to the cell density.

In contrast to the cerebellar cortex, the loss of neurons was dramatically evident at the cerebellar nuclei (Fig. 1, Table 1). The number of neurons in the fastigial nucleus was significantly lower in the 3xTg-AD group, with a mean value 24% that of the NTg mice (p = 0.050). The treatment with scFv-h3D6 allowed for the maintenance of 61% of the cells of the 3xTg-AD compared with the untreated NTg (p = 0.028) and 67% compared with the treated NTg (p = 0.014). No significant difference was found between treated and untreated NTg groups. In any case, it is clear that scFv-h3D6 treatment protected fastigial nucleus neurons from death, although its beneficial effect did not reach non-pathological conditions. Photomicrographs of sagittal sections at the level of the fastigial nucleus show the involvement of these DCN neurons and the pronounced action of scFv-h3D6 on cell viability (Fig. 2).

When cell counts were done in the interpositus nucleus, a similar effect was found (Fig. 1, Table 1). The cell bodies percentage in the 3xTg-AD was 58% that of the NTg (p = 0.014), whereas treatment allowed for the survival of 87% of the cells. This value is not significantly different to the initial cell count (untreated NTg) (p = 0.243) and, in consequence, a complete protection of neurotoxicity could be interpreted. Although the treated NTg group showed higher cell viability that the untreated one (127%), there was no significant difference among these experimental groups (p = 0.114). As a consequence, scFv-h3D6 treatment completely protected interpositus nucleus neurons from death in the 3xTg-AD mice. Photomicrographs of sagittal sections at the level of the interpositus nucleus show the involvement of these DCN neurons and the recovery of cell viability by scFv-h3D6 (Fig. 2).

When the dentate nucleus was considered, no significant effect of the genotype on the number of neurons was observed (Fig. 1, Table 1). In consonance, treatment did not exert an effect in this region.

Several conclusions emerged from these data: (1) the loss of cells in the 3xTg-AD cerebellum depends on the neuronal type examined; (2) macroneuron depletion in the DCN was regionally variable, being greatest in the fastigial nucleus, lesser in the interpositus and negligible in the dentate nucleus; (3) the administration of scFv-h3D6 protected 3xTg-AD DCN neurons from death, as seen five days after injection of a single dose; and (4) although the single injection of 100 μg of scFv-h3D6 completely rescued 3xTg-AD interpositus neurons to the level of the NTg mice, this dose was not sufficient to completely rescue fastigial neurons, which were the most affected initially.

Discussion

We had previously shown that an antibody fragment, the single-chain variable fragment scFv-h3D6, has the ability to prevent the toxicity induced by the Aβ peptide in human neuroblastoma cell cultures. Additionally, we recently demonstrated the benefits of scFv-h3D6 in five month-old female 3xTg-AD animals, which corresponds to early stages of the disease.

The 3xTg-AD mouse brain develops molecular and histological alterations characteristic of AD, following a regional and temporal involvement homologous to humans. Because some studies revealed the involvement of cerebellum in late phases of the disease, study of the disease progression in the 3xTg-AD mouse’s cerebellum is of interest. Other mouse models that are also linked to PS1 mutations showed cerebellar Aβ deposition in the form of neuritic plaques and Purkinje cell loss. A few Aβ plaques were detected in the molecular layer at 6 mo, but loss of synaptic contacts between parallel fibers and dendritic spines of Purkinje cells and degeneration of granule cells, required 12 mo.
mediolateral axis, with the fastigial nucleus the most affected area, followed by the interpositus nucleus, and dentate nucleus not being affected. Although several studies have demonstrated the direct anatomic connection from fastigial nucleus to encephalic regions as amygdala, hippocampus and cerebral cortex,21 as well as the projection through thalamus of interpositus and dentate nucleus to cerebral cortex,22 this is the first time that deep cerebellar nuclei neurons are shown to be affected at early stages of AD progression.

Although cerebellar neurons have been reported to be more resistant to soluble oligomeric Aβ than other neurons in the brain, we wondered when neuronal vulnerability in the 3xTg-AD cerebellum could appear. We focused the study in both the cerebellar cortex and deep nuclei by counting different cellular populations. Plausibly, we did not observe significant differences in the cerebellar cortex because we studied a very early stage of the disease progression. In contrast, the 3xTg-AD cerebellar nuclei showed a gradient of neural loss through the Table 1. Numerical values of the neuronal populations from cerebellar cortex and nuclei (PC, GC and DCN macroneurons) quantification

|                | NTg/−  | NTg/+  | 3xTg-AD/− | 3xTg-AD/+ |
|----------------|--------|--------|-----------|-----------|
| **Purkinje cells** |        |        |           |           |
| Vermis         |        |        |           |           |
| Cell number    | 788.5 ± 20.5 | 748.5 ± 53.9 | 758.3 ± 35.5 | 789.3 ± 36.3 |
| Length         | 27.3 ± 0.5 | 27.1 ± 0.5 | 24.7 ± 1.5  | 28.3 ± 0.9  |
| Cellular density | 28.9 ± 0.5 | 27.5 ± 1.7 | 30.8 ± 1.6  | 27.9 ± 1.1  |
| Paravermis     |        |        |           |           |
| Cell number    | 685.5 ± 58.6 | 725.5 ± 36.9 | 706.3 ± 39.6 | 681.3 ± 16.9 |
| Length         | 24.9 ± 2.1 | 25.1 ± 0.8 | 24.7 ± 1.0  | 26.1 ± 0.6  |
| Cellular density | 27.6 ± 0.3 | 29.0 ± 1.8 | 28.6 ± 0.8  | 26.1 ± 0.6  |
| **Granule cells** |        |        |           |           |
| Vermis         |        |        |           |           |
| Cell number    | 58192 ± 3464 | 56312 ± 178 | 47815 ± 2634 | 56389 ± 2085 |
| Area           | 3.4 ± 0.2  | 3.4 ± 0.0  | 2.9 ± 0.0*  | 3.2 ± 0.1** |
| Cellular density | 17214 ± 237 | 16827 ± 127 | 16608 ± 626  | 17581 ± 513 |
| Paravermis     |        |        |           |           |
| Cell number    | 41946 ± 4850 | 40263 ± 2553 | 42010 ± 4619 | 44609 ± 3625 |
| Area           | 2.8 ± 0.0  | 2.4 ± 0.0  | 2.6 ± 0.1  | 2.7 ± 0.1  |
| Cellular density | 15024 ± 592 | 16807 ± 932 | 16145 ± 650  | 16297 ± 1012 |
| **DCN macroneurons** |        |        |           |           |
| Fastigial      |        |        |           |           |
| Cell number    | 31.89 ± 2.35 | 29.13 ± 2.53 | 7.50 ± 1.44* | 19.46 ± 2.50/** |
| Area           | 0.37 ± 0.04 | 0.35 ± 0.03 | 0.15 ± 0.01* | 0.27 ± 0.03** |
| Cellular density | 88.61 ± 7.98 | 82.96 ± 2.62 | 60.44 ± 1.08* | 71.06 ± 3.76** |
| Interpositus   |        |        |           |           |
| Cell number    | 27.83 ± 2.73 | 35.47 ± 2.53 | 16.25 ± 0.89* | 24.23 ± 2.96** |
| Area           | 0.49 ± 0.05 | 0.57 ± 0.04 | 0.36 ± 0.02* | 0.46 ± 0.02** |
| Cellular density | 57.20 ± 2.26 | 62.81 ± 5.85 | 44.86 ± 1.77* | 56.30 ± 3.11** |
| Dentate        |        |        |           |           |
| Cell number    | 22.89 ± 1.82 | 25.58 ± 1.94 | 22.00 ± 2.18 | 22.25 ± 2.15 |
| Area           | 0.35 ± 0.04 | 0.41 ± 0.01 | 0.37 ± 0.03 | 0.38 ± 0.05 |
| Cellular density | 66.47 ± 2.34 | 62.04 ± 5.26 | 59.39 ± 1.54 | 62.54 ± 10.08 |

Cell number, area (mm²) and density (number of cells/mm²) were quantified. Results are expressed by means ± SEM *significant vs. untreated NTg group (p ≤ 0.05); **significant vs. untreated 3xtg-AD group (p ≤ 0.05). Treated NTg significances vs other groups are omitted as no significant effects were found upon treatment. Significance values were calculated via Mann-Whitney test. Length refers to that of the monolayer (Purkinje Cells Layer) in mm and cellular density refers the number of PC per unit of length.
Even more exciting, however, is the finding that scFv-h3D6 is able to protect 3xTg-AD DCN neurons from death, although the extent of protection was variable. While administration of scFv-h3D6 completely rescued 3xTg-AD interpositus neurons to the non-pathological level of the NTg mice, this dose was not sufficient to completely rescue fastigial neurons, which were initially the most affected ones. It is important to note that, in contrast to other published studies, i.e., intranasal administration of 1.5 mg/ml scFv twice a week for 14 weeks, in the current work 100 μg of scFv-h3D6 were intraperitoneally administered and the effect assessed five days after this low and sole dose.

In conclusion, we described the Aβ-induced death of deep cerebellar nuclei neurons at early stages of the disease progression in the 3xTg-AD mouse model and their rescue by a single, low dose of scFv-h3D6. Further studies increasing dose and using other stages of the disease might provide interesting information about both the therapeutic potential of scFv-h3D6 and the role of the cerebellum in Alzheimer disease.

Materials and Methods

ScFv-h3D6 was recombinantly expressed in E. coli and purified as previously described. Lipopolysaccharides (LPS), the major endotoxins of gram-negative bacteria, were removed from the protein by using Detoxi-Gel Endotoxin Removing columns (Thermo Scientific).

Triple-transgenic 3xTg-AD mouse harboring PS1M146V, APPSwe, and tauP301L transgenes was genetically engineered at the University of California Irvine. Sixteen 5-mo-old females animals from the Spanish colony of homozygous 3xTg-AD and wild-type non-transgenic (NTg) mice were used in the present study. Further studies increasing dose and using other stages of the disease might provide interesting information about both the therapeutic potential of scFv-h3D6 and the role of the cerebellum in Alzheimer disease.

Figure 2. Illustrative photomicrographs of sagittal sections. At the level of the fastigial and interpositus nuclei the involvement of DCN neurons and its protection by scFv-h3D6 is shown. Bar is 50 μm.
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