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Dissecting the role of sortilin receptor signaling in neurodegeneration induced by NGF deprivation

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ABSTRACT

Sortilin is a member of the family of vacuolar protein sorting 10 protein domain receptors which has emerged as a co-receptor in cell death and neurodegeneration processes mediated by proneurotrophins.

Here we tested the possibility that sortilin deficiency interferes with behavioral and neuropathological endpoints in a chronic Nerve Growth factor (NGF)-deprivation model of Alzheimer's disease (AD), the AD10 anti-NGF mouse. AD10 mice show cholinergic deficit, increased APP processing and tau hyperphosphorylation, resulting in behavioral deficits in learning and memory paradigms assessed by novel object recognition and Morris water maze tests. *Sort1*$^{-/-}$ mice were crossed with AD10 anti-NGF mice and the neurodegenerative phenotype was studied. We found that the loss of sortilin partially protected AD10 anti-NGF mice from neurodegeneration. A protective effect was observed on non-spatial memory as assessed by novel object recognition, and histopathologically at the level of Aβ and BFCNs, while the phosphotau increase was unaltered by knocking out sortilin. We suggest that sortilin might be involved in different aspects of neurodegeneration in a complex way, supporting the view that sortilin functions in the CNS are broader than being a co-receptor in proneurotrophin and neurotrophin signaling.

1. Introduction

The neurotrophin NGF is synthesized from a precursor, proNGF, which displays biological activities distinct from those of mature NGF [1]. ProNGF induces neurodegeneration and cell death under acute and chronic situations of the nervous system upon binding to p75NTR and sortilin receptors [2].

We extensively characterized a mouse model [3,4] in which neurodegeneration is achieved by the expression of a recombinant antibody which selectively blocks the activity of mature NGF [5]. We hypothesized that an imbalance of the NGF to proNGF ratio would account for the neurodegeneration observed in the anti-NGF transgenic mice [6,7] and provided supporting evidence by crossing anti-NGF to p75NTR$^{-/-}$ mice [6,7].

In this study, using a similar approach, we explored the role of the pro-neurotrophin co-receptor sortilin in the progression of neurodegeneration in AD10 anti-NGF mice, a line of transgenic mice in which anti-NGF antibodies are obligatorily expressed in lymphocytes and therefore are initially only found in serum and later, after disruption of the blood brain barrier, also in the brain [8]. AD10 mice develop a central neurodegeneration characterized by cholinergic deficit, tau hyperphosphorylation, amyloid β (Aβ) accumulation derived from the altered processing of endogenous mouse APP, and behavioral deficits [8]. AD10 anti-NGF mice were crossed with *Sort1*$^{-/-}$ mice and the neurodegenerative phenotype was studied. We found that visual working memory and cholinergic deficit in the medial septum are fully rescued in aged mice, while β amyloid and hyperphosphorylated tau are mildly, or not at all, affected by sortilin deficiency.

2. Materials and methods

2.1. Animals

Sortilin-deficient mice were engineered by targeted gene deletion of 126 bp from exon 14 and 303 bp of the subsequent intron of *sortilin* [9]. Homozygous *Sort1*$^{-/-}$ mice were crossed with homozygous AD10 anti-NGF mice [8]. Heterozygous AD10×*Sort1* $^{+/-}$ mice from the first crossing were interbred and sex and age matched wild type, AD10, *Sort1*$^{-/-}$ and AD10×*Sort1*$^{-/-}$ mice were used for analysis. Genotype was determined by standard PCR,

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using the oligonucleotides described in Supplementary methods. In total, 117 mice were analyzed in this study across three different age groups: 3 months (WT = 9; AD10 $n = 15$; *Sort1*$^{-/-}$ $n = 10$; AD10×*Sort1*$^{-/-}$ $n = 14$), 6 months (WT = 6; AD10 $n = 11$; *Sort1*$^{-/-}$ $n = 6$; AD10×*Sort1*$^{-/-}$ $n = 12$) and 12 months (WT = 9; AD10 $n = 8$; *Sort1*$^{-/-}$ $n = 6$; AD10×*Sort1*$^{-/-}$ $n = 11$).

2.2. Behavioral tests

Object recognition test was performed over three consecutive days as described [10]. A detailed description of the Morris water maze test has been provided in Supplementary methods.

2.3. Tissue collection and immunohistochemistry

After behavioral analysis, mice were anaesthetized with an excess of 2,2,2-tribromethanol (400 mg/kg) and intracardially perfused with a 4% solution of paraformaldehyde in PBS. Brains were processed for immunohistochemical analysis as described [3,11]. Primary antibodies concentration is provided in Supplementary methods.

2.4. Stereology

Stereological analysis on ChAT-immunoreactive neurons, number of Aβ clusters of dystrophic neurites, AT8-immunoreactive neurons in the hippocampus and lateral entorhinal cortex was performed as described [8,12].

2.5. Statistical analysis

Statistical analysis was performed using the SigmaSTAT program version 3.5 (Systat Software Inc., San Jose, CA). The alpha was set at 0.05 and a normality and equal variance test were first performed. One-way ANOVA or Kruskal Wallis ANOVA was used for multiple comparisons, followed by Bonferroni or Holm-Sidak *post hoc* tests.

3. Results

To determine the contribution of sortilin on the progressive neurodegeneration induced by anti-NGF antibodies, homozygous *Sort1*$^{-/-}$ mice were bred to homozygous AD10 anti-NGF mice and newborns were intercrossed. Littermates with the following genotypes were analyzed: wild type (WT), homozygous sortilin deficient animals (*Sort1*$^{-/-}$), AD10 mice and AD10 mice homozygous for *Sort1 null* alleles (AD10×*Sort1*$^{-/-}$). Mice were examined at 3, 6, and 12 months of age, corresponding to incipient, intermediate and full-blown neurodegeneration in AD10 mice, respectively [8].

3.1. Sortilin loss protects AD10 mice from late non-spatial memory but not from spatial memory deficits

Non-spatial memory was analyzed by the object recognition test. Mice from all genotypes spent the same time in exploring the two identical objects to which they were exposed during the sample phase of the test at all ages examined (Supplementary Fig. 1A–C, respectively at 3, 6 and 12 months of age). In the test phase, at 3 months of age, mice from all genotypes explored more the new object (Supplementary Fig. 1D, $P < 0.05$). At 6 months of age, AD10 displayed the expected memory deficit [8], as they explored equally the new and the familiar object (Fig 1A), while AD10×*Sort1*$^{-/-}$ mice, similarly to WT, and *Sort1*$^{-/-}$ mice, continued to explore more the new object (Fig. 1A, $P < 0.05$). Surprisingly, at 12 months of age the non-spatial memory deficit appeared also in *Sort1*$^{-/-}$ mice (Fig. 1B), while AD10×*Sort1*$^{-/-}$ mice continued to explore more the new object than the old one (Fig. 1B, $P < 0.05$). Next, we explored the possibility that loss of sortilin may also affect spatial memory deficits in wild type and AD10 mice, using the Morris water maze test. Mice from the different genotypes swam with the same speed at all tested ages, with the exception of 6 month-old *Sort1*$^{-/-}$ mice which swam faster than the other groups of mice (Supplementary Fig. 2A, C and E). At 3 and 6 months of age, mice from all genotypes learned to recognize the location of the platform (Supplementary Fig. 2B,D), while at 12 months of age, AD10 mice showed, as previously documented [8], a deficit in learning where the platform was (Fig. 1C, $P < 0.05$). This deficit was partially rescued in AD10×*Sort1*$^{-/-}$ mice (Fig. 1C). At all ages, AD10 mice showed a spatial memory deficit during the probe phase (Fig. 1D–F). *Sort1*$^{-/-}$ mice did not show spatial memory deficits at 3 months of age (Fig. 1D), while an impairment was observed in older mice (Fig. 1E and F, respectively at 6 and 12 months of age). Sortilin deficiency was insufficient to protect AD10 mice from spatial memory deficit, since, at 3 and 12 months of age, AD10×*Sort1*$^{-/-}$ showed an impaired memory of platform location (Fig. 1D and F), while 6 months old AD10×*Sort1*$^{-/-}$ mice showed no spatial memory deficit (Fig. 1E). We conclude that sortilin loss, *per se*, determines memory deficits (Supplementary Table 1), while, in the context of NGF deprivation, it fully protects from non-spatial memory deficits and, in a more limited and time restricted way, from spatial memory deficits.

3.2. Genetic inactivation of Sortilin prevents the increase of Aβ immunoreactive dystrophic neurites in aged AD10 mice

To examine the influence of sortilin gene inactivation on the amyloid pathology in AD10 mice, we evaluated the number of clusters of dystrophic neurites immunoreactive for Aβ/APP, which appear in the 6 months-old AD10 hippocampus [8]. The number of Aβ/APP clusters was not significantly different in 3 months old AD10, *Sort1*$^{-/-}$ or AD10×*Sort1*$^{-/-}$ mice, with respect to WT (Fig. 2A). 6 months old AD10 mice showed the expected increase in the number of Aβ/APP clusters compared to WT (Fig. 2A, $P < 0.05$ and [8]), *Sort1*$^{-/-}$ have the same number of Aβ/APP clusters as WT mice, while AD10×*Sort1*$^{-/-}$ mice showed an equivalent number of Aβ/APP clusters to AD10 mice (Fig. 2A). However, at 12 months of age, sortilin loss in AD10 mice resulted in a twofold decrease in the number of Aβ/APP clusters compared to AD10 mice (Fig. 2A, B and D). Surprisingly, at 12 months, *Sort1*$^{-/-}$ mice showed an equivalent number of Aβ/APP immunoreactive dystrophic neurites to age-matched AD10 mice (Fig. 2A–C). Thus, inactivation of *sortilin* in AD10 mice delays the amyloidogenic process, determining a marked protection at 12 months of age. However, at this age, sortilin loss *per se*, appears to be amyloidogenic.

3.3. Loss of sortilin does not prevent mislocalization and increase of phosphorylated tau

The effects of sortilin loss on phosphorylated tau was analyzed by immunohistochemistry with the phospho-tau specific antibody mAb AT8. In 3 months old AD10 mice, AT8 labels neurons in the lateral entorhinal cortex (LEC) (Fig. 2H, $P < 0.05$ versus WT mice) and, to a lesser extent, in the hippocampus (Supplementary Fig. 3), with a prominent somatodendritic localization (not shown). At the same age, *Sort1*$^{-/-}$ and AD10×*Sort1*$^{-/-}$ mice showed an equivalent number of AT8-immunoreactive neurons in LEC (Fig. 2H), while, in the hippocampus, sortilin deficiency in AD10 mice determined a threefold increase in the number of AT8-immunoreactive neurons (Supplementary Fig. 3). With age, the number of AT8-immunoreactive hippocampal neurons increases in AD10 and, with a delayed time-course, in *Sort1*$^{-/-}$ mice (Fig. 2E, F and