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PRONGF/P75NTR SIGNALING IN HIPPOCAMPAL ADULT NEUROGENESIS: POTENTIAL TARGET FOR GENERATING NEW NEURONS IN ALZHEIMER'S DISEASE

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1. Summary

During recent years, neurogenesis in the adult brain has been well demonstrated in different species, including humans. Interestingly, work with rodents has shown that adult neurogenesis in the dentate gyrus (DG) of the hippocampus is important for some cognitive aspects and that increasing neurogenesis improves memory while its disruption triggers opposite effects. Adult neurogenesis declines with age and it has been suggested that it plays a role in cognitive deterioration and progressive learning and memory loss in Alzheimer's disease (AD). Strategies designed to boost adult neurogenesis may have beneficial effects for the treatment of AD and could counteract the deleterious effects of toxic factors in AD. One of these, the proneurotrophin proNGF, displays a remarkable increase during AD in hippocampus and entorhinal cortex. In contrast to the mature NGF, proNGF has negative functions on survival, proliferation and differentiation. Our hypothesis is that proNGF and its receptor p75NTR play an important role in disrupting adult neurogenesis during AD development and that therefore it can be a promising therapeutic target. However, neither the processes regulated by proNGF/p75NTR during adult neurogenesis nor the cellular and molecular mechanisms involved are completely understood. in this work we use mouse models of AD, AD human samples and primary cultures of NPCs to address the exact role of p75NTR in different aspects of adult neurogenesis including survival, proliferation and differentiation.

2. Results

1. proBDNF expression and the sortilin/p75 ratio are increased in the hippocampus of AD patients

The hippocampus is one of the brain regions most affected in AD. A group of neurons located in the hilus, also known as hilar Mossy cells are particularly vulnerable. We obtained brain samples containing this region from AD patients and controls from the "Bellvitge neurological tissue bank" and using antibodies directed against the extracellular domain of p75 (p75ECD), sortilin (the co-receptor of p75), and the pro-domain of BDNF, we performed immunofluorescence assays to determine the levels of these proteins in the

two groups. We observed that fluorescence intensity for p75 in individual hilar cells of AD brains was not significantly different on average compared to control brains. However, Sortilin fluorescence as well as the percentage of double positive cells for sortilin and p75 increased significantly in AD brains. We also observed an abundant expression of proBDNF in the cytoplasm of the hilar neurons of human hippocampus.

However, in AD brain samples, fluorescence intensity of proBDNF was significantly higher than in control brains. Next we asked whether proBDNF and BDNF could be detected in the CSF of AD patients and controls since this technique has been traditionally used to study the expression profile of different markers in living individuals for the diagnosis and/or prognosis of neurodegenerative diseases. In this case, we analyzed the CSF by western blot and used antibodies against the mature form of BDNF that also detects the proBDNF. Levels of mature BDNF (14 kDa band) in CSF were very low compared to proBDNF (34 kDa band) and required longer exposure times that were shown in a separate panel. Densitometric analysis of the two bands in 15 AD-affected patients and 15 control individuals revealed that the total levels of BDNF (proBDNF+BDNF) were similar between the two groups. However, in AD patients we observed an increase of proBDNF associated with a decrease of BDNF, compared to controls. When we calculated the ratio proBDNF/BDNF in each sample, we observed that the average ratio proBDNF/BDNF was significantly higher in the CSF from AD patients. These results indicate that the CSF is a reliable sample that recapitulates the changes in proBDNF/BDNF expression that take place in the brain of AD patients. In summary, we conclude that hilar neurons of AD patients are more susceptible to cell death due to the higher levels of proBDNF and sortilin expression and the higher proportion of cells expressing both p75 and sortilin.

2. AGE modifications in proBDNF are increased in the hippocampus and the CSF of AD patients

The increase of oxidative stress in the brain with aging has been proposed as a key factor triggering and/or enhancing AD. The molecular mechanisms involved are diverse and include post-translational modifications of specific proteins. For instance, GO and MGO, two highly reactive dicarbonyls that are increased during OS, react with free amino groups of Lys, Arg and Cys residues leading to the formation of the AGE/ALE adducts CML, CEL

and intermolecular crosslink. As we have previously reported (Kichev et al. Am. J. Pathol, 2009), these modifications affect proNGF during AD making the proneurotrophin form of NGF more stable (as it cannot be converted to mature NGF), which in turn increases cell death. To determine whether proBDNF could also be modified by reactive dicarbonyls in AD, we performed different approaches. First, we studied the colocalization of proBDNF and CEL by immunohistochemistry in the hilar region of human hippocampal samples from controls and AD-affected brains. We observed a remarkable increase of CEL immunoreactivity in AD brains compared to controls. Interestingly, almost all the cells in the hilar region of AD patients with significant CEL staining, also co-expressed proBDNF, while in control brains very few neurons displayed double immunoreactivity. These results strongly suggested that proBDNF could be modified by reactive dicarbonyls. In order to address this question directly we performed a more specific assay. We immunoprecipitated proBDNF from the CSF of controls and AD patients and analyzed the precipitates by Western blot with an antibody against CEL. We found that the proBDNF in the CSF from AD patients showed a prominent CEL modification, around sixfold on average, compared to controls. Altogether these results indicate that the proBDNF in AD patients displays AGE post-translational modifications as a consequence of an increase of oxidative stress during neurodegeneration that might prevent the action of convertases to produce mature BDNF.

3. proBDNF modified in vitro by MGO induces neuronal apoptosis and decreases neuron differentiation

In order to test the effects of the AGE modifications of proBDNF induced by oxidative stress conditions on neuron survival and differentiation, we treated recombinant proBDNF in vitro with reactive oxygen species that react with Arg and Cys residues, resulting in the formation of intermolecular modifications and crossovers of the protein. This modified proBDNF (MproBDNF) was used to stimulate primary cultures of neurons along with mature BDNF, unmodified proBDNF as well as an unrelated protein, Bovine Serum Albumin (BSA), modified in the same way as the proBDNF (MBSA), as stimulation controls. In these experiments we used differentiated hippocampal neurons obtained from hippocampal NSCs. At 1 day in vitro (DIV), the neurons started to differentiate but it was between 4 and 7 DIV that the degree of maturation is significant. In longer culture

periods, neurons displayed a complex degree of neuritogenesis and differentiation, mainly in the mBDNF treated wells, which was difficult to quantify. Differentiated neurons expressed doublecortin (DCX), βIIItubulin, SV2, p75, sortilin, TrkB and calretinin. These neurons, however, were negative for parvalbumin and calbindin, indicating that they are young granular differentiated neurons. In control cultures treated with mature BDNF for 6 DIV, neurons increased significantly their differentiation compared to unstimulated cultures, as demonstrated by βIIItubulin staining. Interestingly, control stimulation with proBDNF, without AGE modifications, did not increase apoptotic cell death over basal levels; in contrast, it produced similar effects on neuron differentiation as the mature BDNF. However, stimulation with MproBDNF, produced a significant increase of apoptosis and impaired neuron differentiation. These effects were specific for the AGE modified form of proBDNF (MproBDNF) since they were not observed upon stimulation with MGO modified BSA. These results suggest that proBDNF is quickly converted into the mature form in culture, inducing nearly the same effects as the BDNF, while the MproBDNF, which is more resistant to the action of convertases, is more stable and triggers the adverse effects associated with its pro-apoptotic activity.

4. CSF from AD patients induces neuronal apoptosis through proBDNF/p75

Our results have shown so far that in AD patients there is an increase of proBDNF/BDNF expression ratio as well as an increase of expression of some of the key signaling elements involved in the pro-apoptotic effects of this proneurotrophin. Moreover, we have observed that proBDNF from AD patients displays AGE post-translational modifications as a consequence of the increase of the oxidative stress during neurodegeneration. Finally, our experiments on differentiated neurons suggest that these modifications produce a more stable form of proBDNF, a process which can account for 1) the increased levels of proBDNF in AD brains and CSF and 2) the contribution of proBDNF to the cell death associated with the disease. To evaluate this hypothesis further, we directly evaluated the effects of the proBDNF contained in the CSF of AD patients and controls on differentiated primary neurons, as above. Thus, we stimulated the cultures for 6 DIV with CSF from control and AD patients and stained the neurons with antibodies against p75ICD or with DAPI staining, in order to assess the subcellular localization of the protein by confocal microscopy and the apoptotic cell death, respectively. Control CSF-treated neurons

survived and differentiated normally showing a basal rate of apoptotic cell death of around 10%. A high proportion of these neurons displayed a peripheral distribution of p75 in the soma consistent with the presence of the intact receptor in the plasma membrane. In contrast, cultures treated with ADCSF showed an altered morphology with impaired differentiation and a dramatic increase in the number of apoptotic nuclei (reaching 60%). Moreover, under ADCSF treatment, there was a significant increase in the percentage of neurons with nuclear immunoreactivity for p75, indicating that the 20 kDa ICD fragment was shed and translocated to the nucleus. The CSF from AD patients is known to contain several other factors, such as Aβ, which might be responsible for these neurotoxic effects. To determine the level of participation of proBDNF present in the CSF of AD patients we specifically immunodepleted the CSF samples from proBDNF with specific anti-proBDNF antibodies coupled to sepharose AG beads. Successful depletion was demonstrated by Western blot with antibodies against proBDNF. We then compared the effects of the immunodepleted CSF (CSFID) and the normal CSF from both, AD patients and controls, in our neuron cultures after 6 DIV. Depletion of proBDNF produced a remarkable reduction of the percentage of apoptosis as well as the percentage of neurons with nuclear immunoreactivity for p75. Therefore, these results indicate that the proBDNF in the CSF of AD patients is biologically active and that it is mainly responsible for the pro-apoptotic effects triggered by AD CSF. Moreover, these results strongly suggest that the effects of proBDNF in AD CSF are mediated by activation of p75 signaling.

5. APP/PS1 mutations increase p75 processing and apoptosis induction

In familial AD, one of the most frequent targets of inherited mutations is the presenilin-gamma secretase complex. Some of these mutations affect mainly presenilin 1 (PS1) including the deletion of exon 9 (Δ9) or M146V. These are supposed to be gain of function mutations leading to an increase of protease activity. The study of the pathogenic mechanisms underlying these mutations and the development of AD has been focused on several of the PS1 substrates, mainly Aβ. Since p75 is also a substrate of PS1 activity, we hypothesized that the harmful consequences of proBDNF on neuron survival and differentiation in AD, due to its higher stability as a consequence of AGE modifications, could be boosted by the effects of PS1 mutations on p75 signaling. In particular, we were interested in ascertain whether the shedding of p75, which requires PS1 activity, changes

during AD and whether these changes can sensitize the effects of pro-neurotrophins. To test this hypothesis, we set up primary neuron cultures from NSCs from heterozygous APP/PS1 mice or from wild type littermates as controls. This APP/PS1 animal model has been widely used in studies of neurological disorders of the brain, specifically AD, amyloid plaque formation, and aging. After 6 DIV, when all the conditions are considered for the analysis (regardless of the treatment), APP/PS1 cultures do not show a percentage of apoptosis significantly different from WT. However, under treatment with MBSA (control), these transgenic APP/PS1 neurons displayed a higher percentage of apoptotic nuclei and a higher percentage of neurons with nuclear immunoreactivity for p75 Interestingly, stimulation with MproBDNF induced a stronger and significant increase in cell death and p75 nuclear immunoreactivity in these transgenic neurons compared to controls. We therefore conclude that PS1 mutations involved in familiar AD potentiate the effects of proBDNF on cell death most probably by increasing p75 signaling and, in particular, p75ICD nuclear translocation.

Our results emphasize the important contribution of p75 signaling, including p75ICD internalization and apoptosis, in AD pathogenesis upon AGE-modified proBDNF stimulation. More importantly, our data provide a novel pro-apoptotic mechanism in AD and involving p75 in which higher levels of cleavage due to mutations in PS1 make these neurons more sensitive to the adverse effects of AGE-modified proBDNF. Moreover, we suggest that this is a general mechanism in AD, also for the non-familial, spontaneous form of the disease, given the fact that Aβ is able to trigger p75 cleavage and p75ICD release and could therefore prime p75 signaling for AGE modified proBDNF independently of PS1 mutations.

3. Relevance with possible future implications

Due to the increase in population aging in our area, EA is a major health and economic problem. Unfortunately, there is no specific, successfully recognized treatment for the disease. In this sense, the results from the project indicate that proneurotrophins may be clinically relevant in: a) as inductors of hippocampal apoptosis in the EA; b) as inhibitors

of adult neurogenesis and therefore blockers of episodic memory, mainly of the establishment of the patterns of memories. c) the effect of the cumulative modifications derived from the oxidative stress that affect the proneurotrophins and prevent their processing by aggravating their proapoptotic function; d) early diagnosis of the disease, since we have detected a specific and significant increase in CSF samples of patients affected by the disease.
The results obtained in this work can contribute to the design of therapeutic and preventive methods to treat the disease.

4. Publications

Fleitas C, Piñol-Ripoll G, Marfull P, Rocandio D, Ferrer I, Rampon C, Egea J, Espinet C. proBDNF is modified by advanced glycation end products in Alzheimer's disease and causes neuronal apoptosis by inducing p75 neurotrophin receptor processing.Mol Brain. 2018 Nov 14;11(1):68.

Fleitas C, Ferrer I, Rampon C, Egea J, Espinet C. the lack of p75NTR in vivo ameliorates adult neurogenesis, learning and memory in PS1/APP mice model of Alzheimer’s disease. In preparation

Main Congress Communications

Espinet C, Piñol G, Rampon C, Fleitas C, Peris M, Curià R, Ferran P and EgeaJ.ProNGF/p75NTR signalling in hippocampal adult neurogenesis: potential target for generating new neurons in Alzheimer’s disease. AD/PD 2015. Nice, 2015.

Fleitas C, Rampon C, Curià R, Bernaus E, Egea J and Espinet C. ProNGF/p75/sortilin signaling: potential targets to recover neurogenesis in Alzheimer’s disease. FENS 2016 Copenhagen.

Fleitas C, Rampon C, Aso E, Egea J and Espinet C. ProNGF/p75/sortilin signaling: potential targets to recover neurogenesis in Alzheimer’s disease. Paris ISN 2017.

C. Fleitas, G. Piñol-Ripoll, P. Marfull, D. Rocandio, I. Ferrer, C. Claire, J. Egea and C. Espinet. proBDNF is modified by Radical Oxygen Species in Alzheimer's Disease and causes neuronal apoptosis by inducing p75 neurotrophin receptor processing. Salamanca 2018.NGF Meetings.