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Release of growth factors by neuronal precursor cells as a treatment for diseases with tau pathology

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ABSTRACT

The intraneuronal accumulation of the microtubule-associated protein tau in a hyperphosphorylated state and the extracellular deposit of β-amyloid protein constitute the defining neuropathological signature of Alzheimer's disease, the most common type of dementia in ageing Homo sapiens.
There is accumulating evidence suggesting that transplantation of embryonic and adult-derived neuronal precursor cells (NPCs) has a major role for cell based repair strategies in models of acute and chronic injury. In order to determine whether NPCs could rescue tau-related neuronal cell death NPCs were transplanted into the cortex of transgenic mice expressing human P301S tau protein at 2 months of age and the effect followed 2 and 3 months after transplantation. The results demonstrated that following transplantation mouse NPCs differentiated into astrocytes and exerted a neuroprotective effect. In particular, the expression of ciliary neurotrophic factor and glial cell-derived neurotrophic factor was increased near the transplanted cells. A non-significant increase of brain-derived neurotrophic factor expression was instead found in the area of the cortex where neuronal death was rescued.

Key words
Microtubule • Associated tau protein • Growth factors • Neurodegenerative diseases

Introduction

Neurodegenerative diseases of the brain affect more than 10% of the population over the age of 65. Each year more than 4.5 million people around the world develop dementia (Ferri et al., 2005). The number of dementia patients worldwide is projected to increase from 35.6 million in 2010 to 115.4 million in 2050 (Prince et al., 2009) due to the increase in life span and the absence of a successful cure or prevention strategies, placing an important economic burden on our ageing society. Although Alzheimer's disease (AD) is the most common dementia, Parkinson's disease, dementia with Lewy bodies, and frontotemporal dementia are also quite frequent. These diseases are usually sporadic but a small percentage of cases are familial and usually inherited in an autosomal-dominant manner.

The most common neurodegenerative diseases are characterized by the presence of abnormal filamentous protein inclusions in nerve cells (Lee et al., 2001). In AD these intracellular inclusions are made of the microtubule-associated protein tau in a hyperphosphorylated state. Together with the extracellular β-amyloid deposits, they constitute the defining neuropathological characteristics of AD. Similarly, tau inclusions in the absence of extracellular deposits are the defining neuropathological features of progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), argyro-

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philic grain disease and inherited frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17T) (Goedert and Spillantini, 2006). The identification of mutations in the Tau gene in FTDP-17T (Poorkaj et al., 1998; Hutton et al., 1998; Spillantini et al., 1998) has established that dysfunction or misregulation of tau protein is central to the neurodegenerative process and disorders with tau pathology are now grouped under the name of tauopathies. Furthermore, in AD it is the accumulation and dysfunction of tau that causes cell death and correlates better than APP with the appearance of dementia (Braak et al., 1991).

Despite the knowledge that the presence of misfolded hyperphosphorylated tau is critical for the development of disease, the mechanism of tau-related neuronal cell death is still not understood.

Tau is a neuronal microtubule-associated protein involved in microtubule assembly, stabilization, and axonal transport (Magnani et al., 2007) besides other functions (Ittner et al., 2010). In human brain, there are 6 different tau isoforms that are produced through alternative mRNA splicing of a single gene on chromosome 17 (Goedert et al., 1989). Tau isoforms differ by the presence of 3 or 4 tandem repeats in the carboxy-terminal region and 29 or 58 amino-terminal amino acid inserts. The tandem repeats constitute the microtubule binding domain of tau while the amino-terminal region has been implicated in microtubule spacing, in anchoring to the membrane, and in binding to motor proteins. More than 45 mutations have been described in the Tau gene in cases with FTDP-17T, leading to clinical phenotypes and tau pathology similar to those of sporadic tauopathies, such as PSP, CBD, PiD and AD (Gasparini et al., 2007). One common aspect of all diseases with tau pathology is tau hyperphosphorylation, which reduces binding of tau to microtubules (Lee et al., 2001; Goedert and Spillantini, 2006). Unbound tau accumulates in the cytoplasm up to a critical concentration upon which seeds of aggregation form and, due to nucleation, the small tau oligomers are transformed into filaments that represent the end product of the aggregation process (Maeda et al., 2007). In tauopathies, hyperphosphorylated tau aggregates into filaments that form the inclusions associated with neurodegeneration (Goedert and Spillantini, 2006). Although the oligomers are considered toxic by many, whether they or the filaments are the toxic species is still matter of debate.

The identification of Tau gene mutations has led to the production of transgenic mice expressing human mutant tau as models for tauopathies in which to investigate the mechanisms that link Tau gene mutations to cell death (Lewis et al., 2000). Degeneration of cortical neurons and astrogliosis are the central features of human tauophaties but they have not been consistently reported as features of these experimental models.

There is accumulating evidence suggesting that transplantation of embryonic and adult-derived Neuronal Precursor Cells (NPCs) have a major role for cell based repair strategies in models of acute and chronic injury. Neural precursor stem cells are a population of self-renewing and multipotent cells of both the developing and adult brain that can give rise to the different neuroectodermal lineages of the (Central Nervous System) CNS (Gage, 2000). In several experimental studies exogenous embryonic and adult stem cells have been used with the hope that they could generate new neurons after being transplanted into lesioned nervous tissue. NPCs have the ability to survive after transplantation, migrate specifically within the damaged tissue and maintain their pluripotency. However, there are few data showing the ability of these cells to terminally differentiate into mature neurons and to replace the neuronal function (Cao et al., 2001; Hofstetter et al., 2005; White et al., 2008). Furthermore, it has been suggested that transplanted NPCs might have beneficial effects throught “bystander mechanisms” alternative to cell replacement (Martini and Pluchino, 2006). Thus, NPCs when introduced into a lesioned CNS, can have beneficial effects by providing trophic support to the injured tissue through the production of neurotrophic factors that induce survival and regeneration of host neurons (Hess and Borlongan, 2008).

Against this background the possibility to use NPCs as a potential treatment for tauophaties has been evaluated using a mouse model expressing the human P301S mutant tau protein under the control of the neuron specific mouse Thy-1 promoter (Bugiani et al., 1999; Allen et al., 2002; Delobel et al., 2008; Gasparini et al., 2009). In this transgenic mouse line, abundant neurofibrillary tangles, neuropil threads and microglial activation have been found

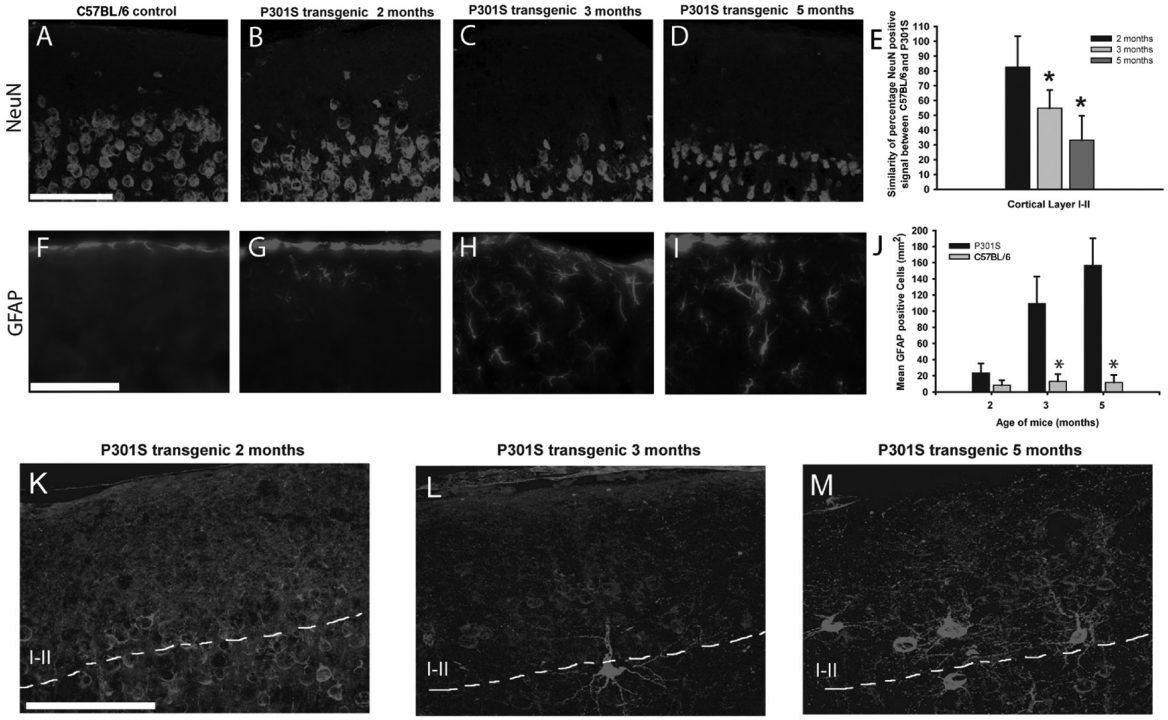

Fig. 1. - Progressive neuron loss (NeuN staining), astrocytosis (GFAP staining) (A-J) and accumulation of hyperphosphorylated tau (AT8 staining) (K-M) in the cerebral cortex of P301S tau transgenic and control mice.

similarly to human tauopathies. The progression of tau pathology as well as behavioral features has been characterized (Scattoni et al., 2010). However, only recently specific cortical areas have been identified where neurons contain ring-like tau deposits in the cytoplasm and progressively die without the appearance of tangles that instead can be present in adjacent cells (Hampton et al., 2010). In this study it has been shown that P301S tau mice have progressive cortical neuronal loss and astrogliosis and transplantation of NPCs or direct NPC-derived astrocytes implantation has a neuroprotective effect.

Materials and methods

Animals

Female and male homozygous P301S tau mice were culled at the onset of symptoms to characterize the neuronal loss (Allen et al., 2002; Bellucci et al., 2004; Delobel et al., 2008; Gasparini et al., 2009) between 4 and 5 months of age, along with age-matched C57BL/6 mice. For subsequent transplantation experiments, both P301S tau and age-matched C57BL/6 male and female mice were culled at 3 and 5 months of age. All procedures were performed in compliance with national and institutional guidelines (UK Animals Scientific Procedures Act 1986 and the University of Cambridge Animal Care Committees).

Immunohystochemistry

Sections for immunofluorescence were processed as described previously (Scott et al., 2005; Hampton et al., 2008). Primary antibodies used were as follows: monoclonal anti-NeuN (1:400, Millipore Bioscience Research Reagents); mouse monoclonal anti-phosphotau (AT8, 1:1000, Autogen Bioclear); monoclonal anti-GFAP (glial fibrillary acidic protein); polyclonal goat or rabbit anti-GFP (green fluorescent protein); rabbit polyclonal anti-BDNF (brain-derived neurotrophic factor) (1:100 Abcam); and rabbit polyclonal anti-GABA (1:500, Sigma). Secondary antibodies used were Alexa 488, Alexa 555, and streptavidin 555 (1:500, Invitrogen) in conjunction with bisbenzamide (1:5000, Sigma) to identify cell nuclei. Cresyl violet (Fisher Scientific) staining was performed by brief immersion into

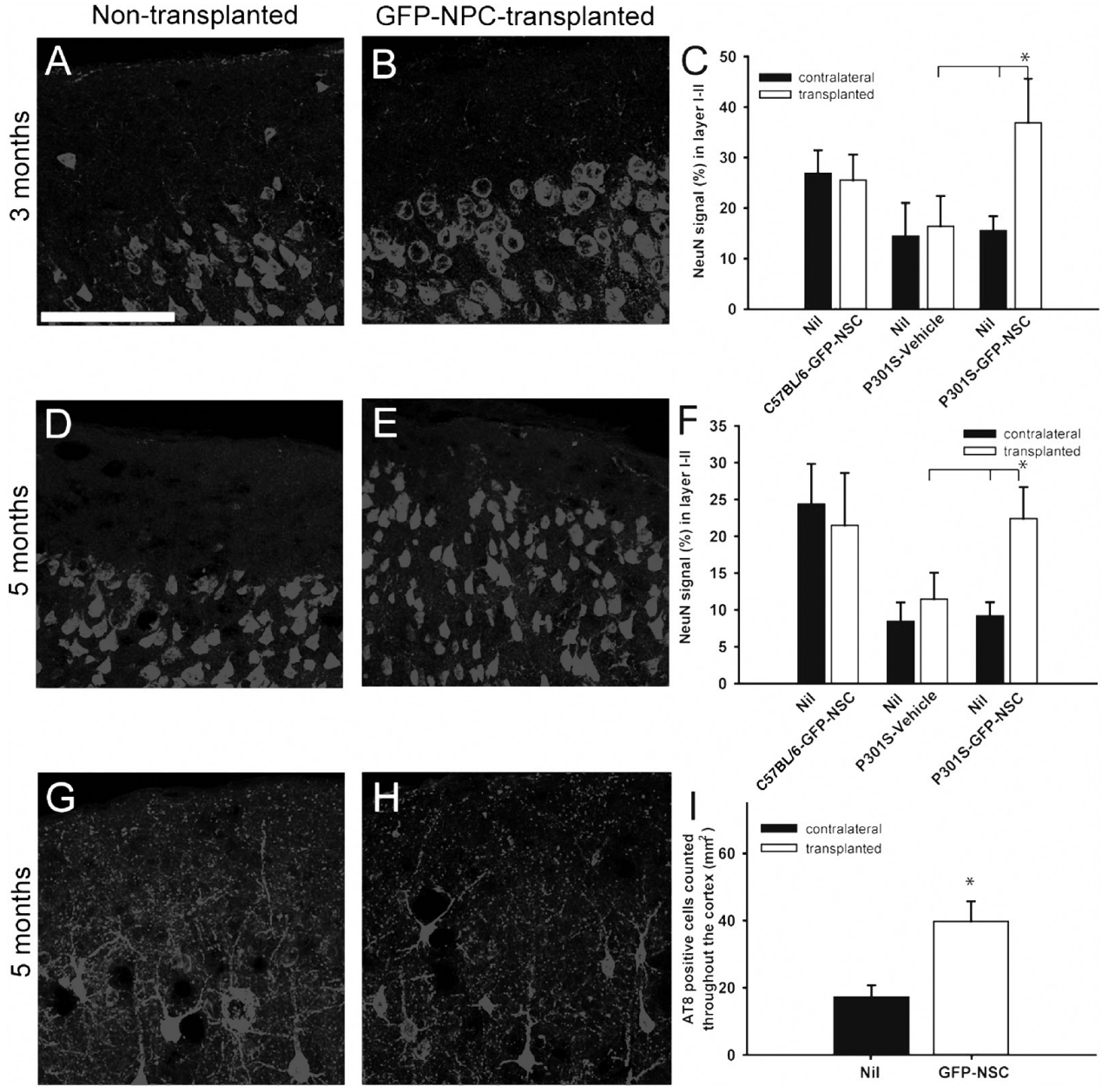

Fig. 2. - Neuroprotective effect of eGFP-NPCs: NeuN staining at 1 (A-C) and 3 (D-F) months after transplantation shows an increase in neuronal numbers; AT8 positive staining (G-I) at 3 months after transplantation shows more neurons with hyperphosphorylated tau deposits.

diluted cresyl violet (0.5%) to identify neuronal cell bodies.

Quantitative analysis

All data were quantified using statistical analysis, including one-way ANOVA (with the Holm-Sidak *post hoc* analysis) or *t*-test, and significance was only assumed if $p < 0.001$.

Transplantation

eGFP-NPCs or eGFP-astrocytes were injected atraumatically into P301S tau transgenic and C57BL/6 male and female mice. Each animal received two injections of eGFP-NPCs (80,000 cells per injection of 0.8 µl) into the cortical gray matter. In experiments parallel to those of the live cell transplants, dead cells and vehicle consisting of the control

media were also injected. Injections were performed in 2 months old animals, which were culled either at 1 month or 3 months after injection. Mice were either perfused with 4% paraformaldehyde or their brains were snap frozen. Perfused brains were cryoprotected in 25% sucrose, frozen, cut using a freezing microtome, and stored at -80°C. Snap-frozen tissues were stored at -80°C and used for quantitative PCR (qPCR).

Quantitative real-time PCR

Total RNA was extracted from tissues microdissected around the injection site using Trizol (Invitrogen) and PureLink RNA mini kit (Invitrogen) according to the manufacturer's protocol. Total RNA (2 μg) was treated with RNase-free DNase I (New England Biolabs) and reverse transcribed in 100 μl with random hexamers using Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was performed in 96-well plates using 1 μl of synthesized cDNA and forward and reverse primers [mouse (m)Reelin forward: 5'-CGAGTGGGTGAGGTGTAT-3'; mReelin reverse: 5'-AGCTATGCTTGACCGTTGCTC-3'; mCux1 forward: 5'-GCGGCGTTCCTGAGTGTTTAT-3';mCux1 reverse: 5'-CTGGCAGGTGGTTACCGTT-3'].

Results

P301S tau transgenic mice characterization

Quantitative neuronal NeuN staining and total cresyl violet staining cell count analysis on cortices from 5 month-old transgenic P301S and C57BL/6 control mice was performed to evaluate cortical neural degeneration. A significative reduction in the percentage of superficial cortical neurons containing NeuN positive cells was observed in the motor cortex of P301S transgenic mice compared to the control mice together with a reduction of GABAergic positive neurons. Quantitative PCR and immunohistochemistry of layer specific markers showed a reduction of Reelin and no significative change of Cux1, respectively markers of superficial and deeper layers. These findings demonstrate that there is a significant neuronal cell loss restricted to the superficial cortex of P301S mice at 5 months of age. Furthermore quantitative analysis of the superficial cortical neurons in 2 and 3 month-old P301S mice showed that cell loss is progressive, starting at 2 months of age. Immunohystochemistry performed using the phosphorylation-dependent anti-tau antibody AT8 in 2 month-old P301S tau mice, showed different tau stainings a ring-like structure in some cells quile in others larger tau aggregates were present in cell bodies and dendrites. The number of cells with tau deposits increased at 3 months and 5 months.

A significative increase of GFAP-positive astrocytes was also observed in the superficial frontal cortex of P301S mice compared with C57BL/6 control from 2 to 5 month-old mice.

These data show that P301S mice have progressive superficial neuronal loss and astrogliosis. Moreover neurons containing ring-like tau deposits in the cytoplasm progressively die before the formation of proper neurofibrillary tangles.

Neuroprotective effect of trasplanted NPCs and astrocytes

To examine whether focal implantation of neuronal precursor cells may influence neuron loss in P301S mice, transplantation studies were performed. NPCs were derived from cortices of neonatal C57BL/6 mice expressing eGFP ubiquitously and were cultured under substrate-free condition in the presence of EGF and FGF-2 (Vescovi et al., 1993). *In vitro* characterization confirmed the neuronal and glial potential of eGFP positive stem cells. Live eGFP-NPCs, dead eGFP-NPCs or vehicle were transplanted into the cortex of 2 month-old P301S mice and age-matched control mice. Brain sections were then analysed at 1 and 3 months after transplantation.

Stereological analysis revealed that around 30% of transplanted eGFP-NPCs were able to survive and integrate around host neurons up to 3 months after transplantation in both P301S and control mice. To examine the potential differentiation of eGFP-NPCs immunohystochemistry performed with neuronal and glial specific markers, showed the presence of only glial cells with no neurons at either 1 month and 3 months after transplantation. A significant increase in cortical neuronal numbers was observed in P301S transgenic mice compared to control mice after transplantation of eGFP-NPCs. Immunohystochemistry using AT8 anti-tau antibody at 3 months after transplantation of eGFP-

NPCs showed more AT8 positive cells with large tau deposits in the superficial cortex where neurons had survived. No cells lshowed both AT8 and eGFP staining. This suggests that transplanted cells do not migrate to substitute those dying. Furthermore, cells that survive accumulate hyperphosphorylated tau and form neurofibrillary tangles that appear increased in the area compared to non-injected P301S tau mice. Furthermore a significant increase in GABAergic neurons as well as Reelin mRNA expression was observed in 1 and 3 month-old P301S tau mice after transplantation.

To test if the neuroprotective effect of NPCs transplantation was dependent from the increase in neurotrophic factors, candidate growth factors were analysed by quantitative PCR. Although, expression of both CNTF and GDNF mRNA was increased in areas containing transplanted eGFP positive cells, only GDNF mRNA was significantly increased compared to controls. Immunohistochemistry and confocal microscopy confirmed these data. Instead no increase in BDNF mRNA expression was observed after transplantation while immunohistochemistry revealed a slight increase of BDNF in the area where cell loss was prevented.

Direct transplantation of differentiated eGFP-astrocytes was then perfomed in order to understand whether the neuroprotective effect was NPCs or glial dependent. eGFP-astrocytes were able to survive and integrate into the host tissue without differentiating into neurons. Following astrocytes transplantation NeuN analysis showed a significant increase in neuronal numbers in the cortex indicating a neuroprotective effect astrocytic-dependent.

Discussion

The present study has shown that mouse NPCs and astrocytes transplanted into the cortex of a mouse model of tau-related neurodegeneration have a neuroprotective effect.

Intracellular inclusions made of hyperphosphorylated tau protein are characteristic of several neurodegenerative diseases including AD and FTD. The finding that Tau gene mutations cause familiar forms of FTDP-17T has established that dysfunction of tau protein leads to the neurodegenerative process (Goedert and Spillantini, 2006) and disorders associated with tau pathology are grouped under the name of tauophaties.

Several transgenic mouse models expressing human mutant tau in neurons and exhibiting the features of tauophaties have been generated. A line of transgenic mice expressing human P301S tau protein under the control of the neuron specific mouse Thy-1 promoter (Allen et al., 2002) was produced in collaboration with Dr Michel Goedert. Four families have been found around the world with the P301S Tau gene mutation and, in general, patients present at an early age with frontotemporal dementia-like phenotype (Gasparini et al., 2007; Bugiani et al., 1999). P301S tau transgenic mice exhibit profuse tau pathology that is most abundant in brainstem and spinal cord where, in homozygous mice, it is associated with 49% motorneuron loss at the age of 5-6 months, when they develop a motor phenotype characterized by paraparesis, tremor, muscle weakness and eye inflammation (Allen et al., 2002; Gasparini et al., 2007). A similar phenotype is present in 12-14 month old heterozygous P301S tau mice.

Similarly to human FTD, P301S mice have progressive, superficial neuronal loss with associated astrogliosis in the superficial layers of the cerebral cortex (Rosso et al., 2001; Broe et al., 2004; Leverenz et al., 2007; Mackenzie et al., 2008). Astrocytosis together with tau dysfunction may play an important role for the progression of FTD (Broe et al., 2004; Kersaitis et al., 2004; Tan et al., 2005). It has been shown that cortical astrogliosis appears to be related to neuronal loss and tau pathology suggesting a role for astroglia in the neurodegenerative process (Broe et al., 2004; Kersaitis et al., 2004).

Furthermore, a proportion of neurons containing ring-like tau deposits in the cytoplasm die before the formation of abundant tau filamentous inclusions.

Previous studies have shown that there are a variety of mechanisms involved in neuroprotection ranging from cell replacement to growth factor mediated functional recovery.

The administration of neurotrophic factors has been used as an efficient protection for suffering neurons in several models of neurodegenerative disorders like insulin growth factor 1 (IGF1) in ALS mice (Lu et al., 2003), glial derived neurotrophic factor (GDNF) in Parkinson's rats and ALS mice (Oliviera et al., 2005; Sugaya et al., 2005), vascular endothelial growth factor (VEGF) in ALS mice and rats (Tanna et al., 2005;

Furmans et al., 2004). The main problem related to the use of these factors is that their distribution into the nervous parenchyma is limited as a consequence of their restricted diffusion across the blood-brain barrier and their relatively short half life. For this reason several approaches have been developed to ensure the production and/or the delivery of growth factors in the site of lesions such as adenovirus, infusion pumps or cellular vectors. Genetically modified NPCs secreting growth factors have also been used as an alternative approach to deliver neurotrophin to a specific brain region following transplantation (White RE et al., 2008). However there are now a lot of evidence suggesting that also non-modified NPCs are able to secrete constitutively a large number of growth factors. The transplantation of NPCs at the site of neuronal degeneration is a promising therapy also for Alzheimer's disease (Gary et al., 1996; Acsadi et al., 2002; Hess and Borlongan, 2008). It has been reported that NPCs injection in a triple transgenic mouse model of Alzheimer's disease improves spatial learning and memory deficits through increased production of BDNF expression (Blurton-Jones et al., 2009).

In the current study the transplantation of embryonic GFP positive NPCs induces an increase in neurotrophin, particulary GDNF and CNTF in the transplant site and possibly BDNF in the site where cell loss is prevented. The eGFP NPCs seemed to have differentiated in situ into glia after transplantation, in particular into astrocytes. To address whether the neuroprotective effect is glia-dependent eGFP positive NPC-derived astrocytes were transplanted into P301S tau mice showing that the neuroprotective effect was maintained. This finding is in line with recent studies reporting an astroglia-mediated neuroprotective effect in different models of neuronal injury (Davies et al., 2008; Lepore et al., 2008; Boucherie et al., 2009). Another potential mechanism for the neuroprotective effect of both the glia-derived NPC transplant and directly injected astrocytes may be through the secretion of Activity Dependent Neuroprotective Protein (ADNP) (Furman et al., 2004; Shiryaev et al., 2009).

In summary this study rconcludes that NPCs and astrocytes have a potent neuroprotective effect in a cortical neurodegenerative disease caused by dysfunction of tau protein. These findings are important to understand the mechanisms of glia-mediated neuroprotection and for the development of future therapeutic strategies for neurodegenerative disorders.

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