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Compromise of cortical proNGF maturation causes selective retrograde atrophy in cholinergic nucleus basalis neurons

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ABSTRACT

The degeneration of basal forebrain cholinergic neurons (BFCNs) in Alzheimer's disease (AD) contributes to cognitive impairment. Nerve growth factor (NGF) secreted in the cerebral cortex is necessary for the phenotypic maintenance of BFCNs. AD is associated with disturbances in NGF metabolism, leading to reduced mature NGF levels and to an accumulation of its precursor, proNGF. We previously described that, in rats, this neurotrophic imbalance is sufficient to induce a loss of cortical cholinergic synapses. In the present study, we investigated whether this neurotrophic imbalance can produce an AD-like retrograde degeneration of BFCNs. Using a combination of retrograde labeling and quantitative cell imaging, we could demonstrate that inhibiting cortical proNGF maturation results in an atrophy of BFCNs, a downregulation of the NGF receptors p75 neurotrophin receptor and tropomyosin receptor kinase A, and a reduction in choline acetyltransferase protein expression. The transient increase in sortilin levels and the sustained colocalization with p75 neurotrophin receptor suggest a participation of proNGF in this degenerative process. This study demonstrates that impairments in the extracellular maturation of proNGF are sufficient to cause a somatodendritic retrograde degeneration of the BFCNs.

1. Introduction

Basal forebrain cholinergic neurons (BFCNs) project axons to the cerebral cortex where they form the most of its cholinergic synapses (McKinney et al., 1983). Nerve growth factor (NGF) is a target-derived neurotrophin necessary for the phenotypic maintenance of adult BFCNs (Cuello, 1996; Debeir et al., 1999; Hefti and Weiner, 1986). As a degeneration of BFCNs contributes to the cognitive symptoms associated with Alzheimer's disease (AD) (Coyle et al., 1983; Davies and Maloney, 1976; Perry et al., 1978; Whitehouse et al., 1981), it was initially suspected that a trophic factor compromise would lead to an atrophy of BFCNs in this condition (Hefti and Weiner, 1986). However, no evidence of altered NGF synthesis could be found in AD patients (Fahnestock et al., 1996; Goedert et al., 1986; Jetté et al., 1994). Because early immunological techniques were unable to distinguish NGF from its precursor, an increase in NGF was reported (Crutcher et al., 1993). Later, it was found that this was caused by increased levels of the NGF precursor (proNGF) (Bruno et al., 2009a; Peng et al., 2004). Importantly, we have previously demonstrated that, in the central nervous system, it is proNGF and not mature NGF (mNGF) that is released by neurons to the extracellular space in an activity-dependent manner, along with the proteases necessary for its maturation and degradation (Bruno and Cuello, 2006), including plasmin, the enzyme that converts proNGF to its mature form. Furthermore, in AD brains, we found a compromise in the conversion of proNGF to mNGF, along with an elevation of the mNGF-degrading protease matrix metalloproteinase 9 (Bruno et al., 2009b). Interestingly, the intracerebral injection of beta amyloid oligomers, which are molecules central to the AD pathology, is sufficient to trigger proNGF and matrix metalloproteinase 9 upregulation (Bruno et al., 2009a). More recently, we demonstrated that a very similar deregulation of the NGF metabolic pathway occurs in individuals with Down syndrome (DS) and an AD-like amyloid pathology (Iulita et al., 2014b). Importantly, we have provided some evidence suggesting that changes in plasma levels of NGF-related markers and inflammatory mediators, in combination with cognitive assessments, should allow the identification of individuals with DS likely to convert to AD (Iulita et al., 2016b). The involvement of the NGF

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metabolic pathway in AD and DS has been recently reviewed in (Iulita et al., 2016a; Iulita and Cuello, 2014, 2016).

To validate the impact of these alterations on cholinergic degeneration, we previously used a continuous infusion of α_2-antiplasmin, the endogenous inhibitor of plasmin, to prevent proNGF maturation in the cortex of adult rats and induce an AD/DS-like neurotrophic imbalance (Allard et al., 2012). This manipulation altered cortical mNGF/proNGF ratios and was sufficient to cause a local loss of cholinergic synapses concomitant with impairments in the consolidation of a spatial memory (Allard et al., 2012). This finding indicates that such alterations in the metabolism of NGF can participate in disease progression and recapitulate aspects of AD disease progression.

In AD, however, cholinergic degeneration is not limited to cortical synaptic loss. Cholinergic cell bodies in the basal forebrain atrophy and display altered gene expression (Counts and Mufson, 2005; Counts et al., 2004; Ginsberg et al., 2006; Mufson et al., 1989b, 2000; Whitehouse et al., 1981). The finding that cortical stroke-like lesions in rats result in the atrophy of BFCN cell bodies led to the proposition that such changes could also occur in AD (Cuello and Sofroniew, 1984; Pearson and Powell, 1987; Sofroniew et al., 1983). Importantly, in this model, cholinergic degeneration can be prevented by the timely application of exogenous mNGF (Cuello, 1996; Garofalo et al., 1992; Hefti, 1986; Hefti et al., 1984; Kromer, 1987; Williams et al., 1986). In this report, we coinfused α_2-antiplasmin in the cortex of adult rats, as performed previously (Allard et al., 2012), with the retrograde marker fluorogold. Using this strategy, we could identify the BFCNs that project to the infused area and quantify their expression of cholinergic markers. We noted expression changes after 2 and 4 weeks of infusion, and a significant reduction in cell body size after 4 weeks of infusion. Our findings further validate in vivo the significance of the extracellular NGF metabolic pathway for the phenotypic maintenance of BFCNs. This further supports the concept of a retrograde degeneration of cholinergic neurons in the context of amyloid-induced cortical NGF dysmetabolism.

2. Materials and methods

A total of nineteen 4-month-old Wistar rats were used for these experiments. Experimental groups were distributed as follows: (1) 3 received vehicle + fluorogold for 2 weeks; (2) 3 received vehicle + fluorogold for 4 weeks; (3) 8 received α_2-antiplasmin + fluorogold for 2 weeks; (4) 5 received α_2-antiplasmin + fluorogold for 4 weeks. All procedures were performed in accordance with the McGill Animal Care Committee.

2.1. Surgery

Unilateral cannulas for osmotic pumps (Plastics One, Roanoke, VA, USA cut 1.5 mm below pedestal) were connected by vinyl tubing to osmotic pumps (Alzet, Cupertino, CA, USA, models 2002 and 2004 for 2 weeks or 4 weeks treatments, respectively) that were filled with different solutions under sterile conditions. The pumps contained either fluorogold (0.005%, Fluorochrome, Denver, CO, USA) in saline (0.05 M $NaC_2H_3O_2$ [EMD, Gibbstown, NJ. USA] and 0.1 M NaCl [EMD, Gibbstown, NJ, USA]) or fluorogold and α_2-antiplasmin (Molecular Innovations, Novi, MI, USA) that was dissolved in saline to a concentration of either 0.31 μg/μl for the 2-week treatment, or 0.62 μg/μl for the 4-week treatment. These different concentrations of α_2-antiplasmin were used to compensate for the different pumping rates of osmotic pump models 2002 and 2004. All solutions were sterilized using a 0.2 μm syringe filter (Nalgene, Rochester, NY, USA), and the pumps were filled under sterile conditions in a bio-safety cabinet. Before surgery, the animals were anesthetized with isoflurane, put in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA) and kept deeply anesthetized with the use of a nose adapter for isoflurane anesthesia (Harvard Apparatus, Holliston, MA, USA). Details about the surgical procedure are provided elsewhere (Hu et al., 1997). The cannulas (Plastics One, Roanoke, VA, USA) were implanted at the following coordinates: antero-posterior +0.7, lateral +1.5 relative to bregma, and reached a depth of 1.5 mm below the skull to target caudal end of the frontal cortex. All coordinates were derived from the Paxinos and Watson rat brain atlas, sixth edition (Paxinos and Watson, 2006).

2.2. Immunohistochemistry

After the infusion period, animals were perfused transcardially with 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, their brains were harvested, were postfixed for 2 hours in the same fixative at 4 °C, and were immersed in a cryoprotectant solution (30% sucrose in 0.1 M phosphate buffer) for 48 hours. The ipsilateral hemisphere was cut in the coronal plane at a thickness of 40 μm using a cryostat. Sections containing the basal forebrain were collected at regular intervals, for a total of 10 sections per animal for each labeling group. The sections were methodically selected and separated, to ensure that sections from all labeling groups had an equivalent distribution along the anterior-posterior axis.

Selected sections were washed in phosphate-buffered saline (PBS), incubated in a 50% EtOH in ddH_2O for 30 minutes, washed in PBS, and then incubated in PBS + 0.2% Triton X-100 (PBS+T) with 5% normal goat serum (NGS) (Sigma-Aldrich) for 1 hour. Following this blocking step, the sections were incubated overnight at 4 °C in a solution containing the primary antibodies with 5% NGS in PBS+T. The sections were then washed in PBS and incubated overnight in a solution containing the secondary antibodies with 5% NGS in PBS. The sections were washed in PBS, mounted on gelatin-subbed slides, and coverslipped with Aqua Polymount (Polysciences, VWR, Mont-Royal, QC). References on the primary antibodies used can be found in Table 1. To control for any signal not resulting from primary antibody binding, sections were stained following the same protocol, except primary antibodies were omitted. To control for cross-reactivity of secondary antibodies, sections were stained with a single primary antibody on day 1, and all secondary antibodies were used on day 2. This control was done for all primary antibodies used, and signal was consistently exclusive to the detection/imaging channel corresponding to the appropriate secondary antibody, as expected (see Table 2). Bleed-through or cross-reactivity of secondary antibodies would result in signal across channels. For all control experiments, adjustments related to laser settings, filter sets, and detector gain were the same as for quantification in experimental tissue.

2.3. Confocal microscopy and quantification

Images were taken on a Zeiss LSM 510 confocal microscope equipped with Ar and He-Ne lasers (Zeiss Canada, North York, ON) and a titanium sapphire 2 photon laser (Coherent Inc, Santa Clara, CA, USA). The micrographs were acquired using a 63X Plan-Apochromatic oil-immersion objective and the Zeiss LSM software (Zeiss Canada, North York, ON). For each of the 19 rats included in the study, 10 sections per immunolabel were sampled, and 5 to 10 images were taken per section. For each rat, an average of ~35 fluorogold positive cholinergic neurons was quantified from the choline acetyltransferase (ChAT)/p75 neurotrophin receptor (p75NTR)/tropomyosin receptor kinase A (TrkA) triple labeling, and another ~35 were quantified for the ChAT/p75NTR/Sortilin labeling. An average of ~15 fluorogold positive noncholinergic neurons and ~30 fluorogold-negative cholinergic neurons were quantified per animal for cell body size. Fewer fluorogold positive noncholinergic neurons were used for analysis because of their lower